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Marshall R. Urist, M.D., Guest Editor



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Diseases of the Hip in Children, 1962
Disorders of the Cervical Spine, 1962

All contributors desiring to submit articles for consideration for publication on the topics listed above or in the general sections of this publication should submit them to the editor some months in advance of the date of the issue for which they are intended

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Section I

CLINICAL PHYSIOLOGY AND PATHOLOGY OF BONE

An Issue Published As a Part of the Celebration of the 72nd Birthday of Franklin Chambers McLean, M.D., Ph.D.

MARSHALL R. URIST, M.D.
GUEST EDITOR

GREETINGS
FROM PUPILS, COLLABORATORS AND CORRESPONDENTS OF
FRANKLIN C. McLEAN
ON HIS 72ND BIRTHDAY

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   Paul Hodges
2. Lemont, Illinois
   Argonne National Lab... Leo Marinelli
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24. Dunedin, New Zealand
   University of Otago... W. Colin Durning

Preface

Marshall R. Urist, M.D.

The most important change in the field of orthopaedic surgery in the past 10 years is the trend toward study of the patient as an individual and the conditions that cause musculoskeletal disease and their relation to the function, the deformity and the future disability. The importance of continuous experimental investigation and reinvestigation of normal and pathologic physiology of injury and disease of connective tissue is now well established. The articles in pages to follow deal with or bear on the problems of musculoskeletal disease in animals and in man, and have a direct bearing on orthopaedics in the broad sense of the word. The authors—all associates, collaborators, correspondents or pupils of Franklin C. McLean—represent every known discipline or specialty of biology and medicine, and are distributed throughout 14 different nations of the world (Treasure Map, p. 2). They emphasize the importance of bone and joint disorders, whether they are observed in man or produced experimentally in animals. The object is to publish an issue of *Clinical Orthopaedics* that reflects the area of this specialty as broad as Franklin McLean predicted that it would become in the future, in a lecture presented 12 years ago to 2,000 orthopaedic surgeons. He said:

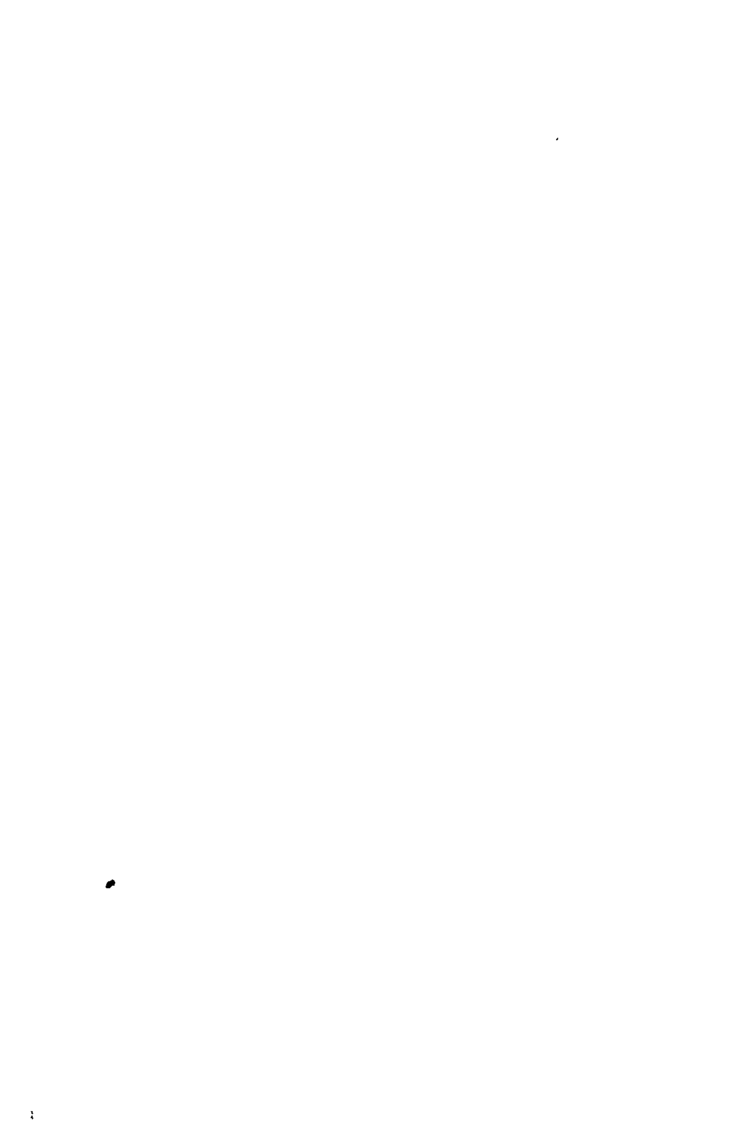
Who is to accept the challenge of nontraumatic disorders of the skeleton? And my answer is that you will. And you will for the simple reason that in the long run no one will know more about the bones and their idiosyncrasies than you will. Wherever you are in practice, you will find that these problems are laid on your doorsteps, and that your success in practice will be measured not only in terms of your ability to deal with injury and deformity, but equally in terms of your grasp of the metabolic and systemic problems that come to you.*

The contributors to this issue are scientists working on the frontier of the field of bone To communicate with one another, the most effective instruments are the Josiah Macy, Jr. and Gordon Conferences, and special publications such as this one. In this way, a large number of investigators contribute to an international pool of observations and work as a team to produce new knowledge for possible later applications to orthopaedics.

The publication of this volume was supported in part by grants-in-aid from the Lee Mytinger Foundation for Medical Research and from Ayerst Laboratories, Inc.

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* McLean, F. C: Physiology of bone, in lectures on regional orthopedic problems, *in* Am. Acad. Orthop. Surgeons, Lect. 2:21-23, 1948.



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Aliis Aedificat*

Paul C. Hodges, M.D.†

One man—Franklin C. McLean—mapped the broad operational plan of the medical school, clinics, hospital and staff of the University of Chicago. Presidents, trustees, members of the Rockefeller boards, dedicated faculty members, contributors of money ranging from a few dollars to millions, have played an essential and greatly appreciated role. But one man above all others developed the best-organized and most frequently admired institution of full-time medicine known in the United States. This volume, published as part of the festivities to celebrate Dr. McLean's 72nd birthday, should contain his curriculum vitae and the story behind the design of his bookplate (p. 6).

Born on February 29, 1888, in Maroa, Ill., the son and the grandson of physicians, Dr. McLean received a B.Sc. degree from the University of Chicago when he was only 19 years old and was elected to Phi Beta Kappa the same year. During medical school days he served as assistant in pharmacology, and, after receiving his M.D. from Rush in 1910 and taking an internship at Cook County Hospital, he was called to the University of Oregon as Professor of Pharmacology. During the course of the Oregon appointment he came back to Chicago for a Summer Quarter as instructor in pharmacology, was awarded an M.S. degree, and subsequently studied for 8 months at the universities of Graz and Vienna (1912-1913).

He spent the period 1914 to 1916 at the Rockefeller Institute, working with Donald Van Slyke and Alfred E. Cohn; there was a Ph.D. in physiology from the University of Chicago in 1915; and in 1916 Dr. McLean accepted the directorship and the chairmanship of medicine in the medical school that the China Medical Board of the Rockefeller Foundation was preparing to build in Peking.

A journey to China in the summer of 1916 was the first of many trips to the Orient during the planning, building and early operation of the Peking Union Medical College; and, in addition to the China responsibilities, Dr. McLean served as first lieutenant, captain and major in the Medical Corps of the United States Army between 1917 and 1919. With the close of World War I he took up residence in Peking, resigning the directorship so that he might devote all his time to the chairmanship of the Department of Medicine. In the spring of 1923, after marrying Dr. Helen Vincent, he returned to the United States and became chairman of the newly created Department of Medicine at the University of Chicago.

For the next 5 years, Dr. McLean served the University of Chicago in a capacity that many institutions dignify with the title "Vice-President in Charge of Medical Affairs," but until he was made Director of the University Clinics in 1928 his only official status was that of Professor and Chairman of the Department of Medicine. In the

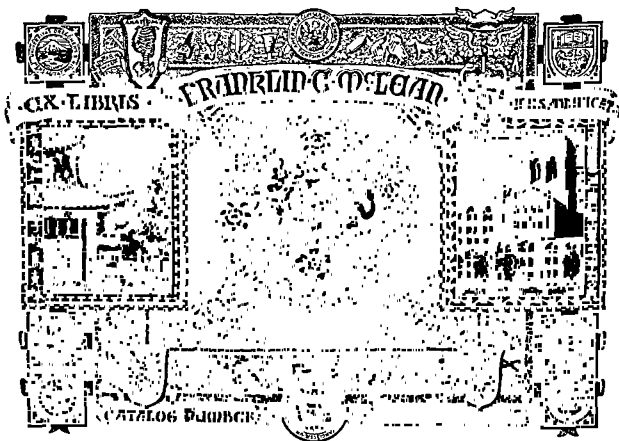
* Published with the permission of the Bull M. Alumni A., University of Chicago, Winter Issue, 1954

† Department of Radiology, University of Chicago, Division of Biological Sciences, School of Medicine, Chicago 37, Ill

9 years from 1923 to 1932 the physical nature and intellectual character of the medical school at the University of Chicago assumed the pattern that seems so reasonable and inevitable to us today, and the "full-time" principle, which at first had few advocates and innumerable critics, became so firmly established that it was able to survive the passage of the nation from the easygoing affluence of the 1920's to the financial depression of the 1930's. Growth of plant and staff was all but stopped at the depth of the depression, but, with the easing of financial stringency in the late 1930's, that growth was resumed, and in recent years it has been greatly accelerated.

In 1933 Dr. McLean resigned from clinical and administrative work to become Professor of Pathological Physiology in the Department of Physiology, and the succeeding 20 years proved to be more scientifically productive than any previous period in his life. Several scores of publications record the work of this period, most of them concerned with some phase of mineral metabolism or the growth of bone. World War II brought service as lieutenant colonel and then colonel in the Medical Corps of the Army of the United States with assignment to chemical warfare service; and during and since the war Dr. McLean has been director of several government projects on the campus and elsewhere, with appointments as "consultant," "panel member," "committee member," etc., too numerous for detailed inclusion here. He holds many military and civilian decorations and citations, and has taken a prominent part locally and nationally in improving facilities for the education of Negro nurses and physicians.

At the time of Dr. McLean's retirement from clinical work in 1933 his colleagues presented him with the bookplate that is reproduced here. It is in the form of a Chinese rug with border ornaments and adjacent plaques picturing the highlights of a



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Franklin C. McLean

1908 –

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3

The McLean Campaigns for Full-Time Academic Medicine*

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The gathering of a great doctor's associates, correspondents, collaborators and pupils to celebrate his birthday and exchange greetings is a most pleasant occasion. Because Dr. McLean will appreciate a scientific project almost as much as the sentiment of the celebration of his birthday, this volume was compiled to present the newer knowledge of bone as a living tissue to a clinical audience. Franklin C. McLean's own contributions to medical science, listed in his bibliography, are interesting as well as informative reading material and cover a broad area of modern medicine. While he is well known for his work in basic science, the impact of his research and the campaigns that he has fought for improvement of education in clinical medicine will be emphasized here. At this time, when the economic structure of many of the academic institutions of the United States rests on tenuous foundations, McLean's experiences in and contributions to full-time medicine are interesting and important to review.

McLean's academic campaigns should not be confused with the world wars in which he served and the government projects in which he still serves his country; the United States was at war before, during and after the many important academic events in which he played a leading part. In this chapter, McLean's academic activities will be termed *campaigns*, and the military events will be referred to as *wars*.

EARLY INFLUENCES AND THE AFTERMATH

Following the reform movement in medicine in the United States during the early twentieth century, Franklin McLean was appointed, when he was a young man, to serve as a front-line field general. The great leaders in scientific medicine of that era—Doctors Rufus Cole, Donald D. Van Slyke, Alfred E. Cohn and Simon Flexner, Mr. Abraham Flexner and others—recruited him and saw him through basic training in the United States and abroad during the period from 1914 to 1925. The broad operational plan is described in Flexner's famous report on medical education, and a first objective was to institute the full-time system in a large American university medical school.

EDUCATION

The great scholars who showed McLean how to use his talents merit careful analysis. Anton J. Carlson, one of America's foremost physiologists of all time, was his first source of stimulation and guidance. When McLean was 19 years of age, Carlson taught him the procedure of performing an experiment and writing a scientific article. Young McLean's

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teachers included one of the finest collection of scientists to gather together in one community: F. R. Lillie (zoology), C. J. Herrick (neurology), R. R. Bensley (anatomy), H. G. Wells (pathology), E. S. Jordan (bacteriology) W. Koch (pharmacology) and others. There was also invaluable postgraduate work under the supervision of Prof. Otto Loewi, of Graz, Austria, in 1913, and Doctors Donald D. Van Slyke and Alfred E. Cohn later in 1914 at the Rockefeller Institute. Dr. Cohn, who presided at the Journal Club meetings, recognized McLean's ability and engaged him in a correspondence about medical education that continued for 42 years!

BASIC TRAINING

In 1916, when Franklin McLean was 28 years of age, he gained experience in an action that was preliminary to the great Chicago Campaign that was to come in 1923. The maneuver area was Peking Union Medical College, where McLean served as director and professor of medicine. G. Canby Robinson, in his book entitled *Adventures in Medical Education,* describes the incident in which young Dr. McLean was interviewed for the job of designing the prototype of an ideal institution of university medicine. One day Simon Flexner, director of the Rockefeller Institute, called McLean in for a "pleasant chat," along with Wallace Buttrick Afterward McLean remarked to a friend, "I've had the once-over for China."[15] There followed the assignment and a long sea voyage to Peking to build the plant and organize the staff of an entire medical school and hospital

The architects under contract to plan the buildings were located in Baltimore. Franklin McLean returned to the United States, reported to the Johns Hopkins Medical School, enrolled as a postgraduate fellow and was assigned a small office to be shared with Alan Chesney (who later became dean of the Johns Hopkins Medical School) to work with the architects. During this time McLean had the privilege of meeting and consulting with Dr. William H. Welch. There is no record of the conversations between the great professor of pathology, who organized the Johns Hopkins Medical School and Hospital, and young McLean, but, as will be related further in this chapter, Welch must have looked upon McLean as new hope for the advance that he himself would have liked to make for a complete full-time program of *academic medicine.*

THE EVOLUTION OF FULL-TIME MEDICINE

Many of the leaders in American education before the era of World War I regarded the idea of full-time medicine with suspicion and as a program of limited value of dubious origins. In 1913, when the staff of the Johns Hopkins Hospital was partially reorganized, some of the heads of the major departments were full time. Halsted thought it an excellent scheme to improve teaching and research and to separate from the university men who needed the stimulus of moneymaking to compel them to work.[11] In 1913, Osler wrote to Dr. George Dock, who had accepted a full-time clinical position in St. Louis under the Rockefeller Programme, and expressed a different opinion:

> It would be a very good thing to have a few men at Research Institutes, Cole at Rockefeller for example, devoting all their time to the work, but what I dread is to have a class of clinicians growing up out of touch, and necessarily out of sympathy with the profession and the public. This would be nothing short of a calamity. There are always men of quiet type, like Halsted, who practically live a secluded life; to have faculty made up of Halsteds would be a good thing for science, but a very bad thing for the profession [6]

On December 4, 1913, before the Abernathian Society at St. Bartholomew's Hospital, Osler recommended the Johns Hopkins Hospital system of organization, but he saw advantages in a full-time staff and

that nowhere yet had there been a practical trial of it. He then asked the famous question and gave the answer that made history:

> Would there be the danger of the evolution throughout the country of a set of clinical prigs, the boundary of whose horizon would be the laboratory and whose only human interest would be research? I frankly say that I am not in favor of the whole-time clinical teacher.[7]

However, at another place in this address, Osler went on to say:

> At the same time, let me freely confess that I mistrust my own judgment, as this is a problem for young men and for the future.[7]

When Harvey Cushing included these remarks on the full-time system at length in the 1926 Pulitzer Prize biography *The Life of Sir William Osler*, his teacher and devoted friend William H. Welch, of Johns Hopkins, accused him of unduly emphasizing Osler's distrust of the full-time plan in a review of the book for the *Saturday Review of Literature* of November 21, 1925, subsequently given wide circulation as a 26-page pamphlet.[9] Cushing acknowledged the criticism but, with due respect for his teacher, changed the subject rather than argue the point too far. Welch observed that the full-time system should not have been called "The Rockefeller Programme" for it did not originate with any Rockefeller board.[9] Actually, it originated with Welch himself as much as any one individual. According to Flexner and Flexner (whose analysis will be condensed as follows), it all began in the nineteenth century when some professors in Germany began voluntarily to devote their whole time to teaching and studying in clinics. In 1873, Hugo von Ziemssen established the first scientific medical laboratory at Erlangen University in Bavaria. The next year he was called to Munich, where in 1884 there was established the first hospital laboratory in which investigators and students worked side by side. Welch saw it in operation during its first year and regarded it as the first modern medical clinic.[8] Welch was intelligent enough to see that he must resist the temptation to advocate this "new medicine" publicly in Baltimore in the Gay Nineties. But he began to work cautiously by stimulating younger professors to try to improve research at the bedside and raise the level of clinical instruction to the standard of basic medical science. His most enthusiastic collaborator was Franklin P. Mall. Mall had already been indoctrinated by Prof. Karl Friedrich Wilhelm Ludwig, of Leipzig, when from 1885 to 1886 he was one of the American pupils to whom Ludwig expounded his ideas on clinical reform.[7,18,19] Mall became professor of anatomy at the University of Chicago 6 years later, in 1892. Flexner and Flexner observed that

> the American history of the modern clinical reform begins with heated talks between Mall, George Vincent, the sociologist who was to become president of the Rockefeller Foundation, and Jacques Loeb, a medically trained biologist whose subsequent scientific work was to be of first importance.[8]

Thirty years before Dr. McLean was to appear on the scene in Chicago to institute the full-time program, Mall had Chicago "resounding with discussions of clinical reform." In 1893, Mall transferred to Hopkins, where he continued to agitate for full-time medicine. Mall's method was intimate conversation with individual doctors.[8] Dr. Lewellys F. Barker (who changed places with Mall as professor of anatomy in Chicago), was Mall's public relations man. He was an inspiring public speaker and, in 1901, declared that professors should work in laboratories adjacent to the wards, should be well paid by the University and, in return, should contribute their fees to the budget of the University. When Welch heard about this, he asked Barker how much of his address was Mall's.[8] *The Journal of the American Medical Association* criticized Barker's views as impractical. However, 3 years previously, in 1898 (29 years before Franklin McLean opened the Univer-

sity of Chicago Clinics), Frank Billings pledged the Rush Medical College to the establishment of a full-time program *whenever funds** could be procured.[8] Welch apparently was one of the few who could appreciate the inflammatory nature of these ideas; as late as 1906, in an address at Harvard, he pointed out the need for scientific medicine but made no reference to "full time." Welch knew this was so expensive as to be prohibitive, but in 1907 he made a speech in Chicago that Flexner and Flexner regarded as a cautious step forward. Franklin McLean was a sophomore in medicine at the University of Chicago when Welch made the following statement that was to have a profound effect on the course of events:

> The heads of the principal clinical departments, particularly medical and surgical, should devote their main energies and time to their hospital work and to teaching and investigating without the necessity of seeking their livelihood in a busy outside practice, and without allowing such practice to become their professional occupation.[8]

The "full-time programme" probably did not originate with any Rockefeller board, but Welch was one of the designers of the full-time staff and program of the Rockefeller Institute for Medical Research. The pattern was the same as the Koch Institute in Berlin and the Pasteur Institute in Paris. The purpose was to promote full-time research, and Dr. Rufus Cole, the first director of the Rockefeller Hospital, aimed at having medicine recognized as an independent science, just as physiology and medicine are independent sciences.[4] This venture was independent of any university. The hospital part of the program was initiated in 1910, 9 years after the Institute was founded. In the same year, Welch appealed to Mr. Rockefeller through the Rev. Frederick T. Gates and Mr. Starr J. Murphy, Mr. Rockefeller's advisers, for finances for a full-time chair (in pediatrics) at Johns Hopkins. He failed to obtain the necessary funds at this

* Author's italics.

time when the interest of the Rocl advisers was in developing the Instit Medical Research. Flexner and Flexne tion a letter from Mr. Gates to stating:

> You have told me some of your troub I want an opportunity to tell you some c and perhaps we can mutually comfoi other.[8]

Three years later, in 1913, when was acting president of the Johns H University, the General Education appropriated funds to employ a limitec ber of full-time men, and the proje named the William H. Welch Endo for Clinical Education and Researcl success of Welch's program dependec the acquisition of medical men wi energy of self-dedication and the willi to make financial sacrifice.

Rufus Cole, now aged 87, writing a on an inquiry of the nature of history McLean's prefatory chapter for the coming issue of *Annual Review of Pl ogy* and commented in a letter datec 20, 1959, that he now believed with ' that

> selection, survival, and evolution take pl response to environmental pressures kinds, including sociological and intell

Dr. Cole looks back on his own past a plishments, as well as those of his asso with extraordinary objectivity in the f ing additional excerpt from the letter:

> What happened was related to the p the successive environments. This doe mean that the men [*like McLean*†] wh outstanding parts were not especially tal but under the circumstances they could hardly acted otherwise than they did.

From all the available evidence it bec clear that the full-time program that Mc instituted at Chicago evolved from the s tific clinics of medicine at Erlangen, Mu and other universities in Germany; the

† Author's addition.

of Welch and several of his younger colleagues, notably Mall, Barker and others at Johns Hopkins; the Abraham Flexner Report of 1910; the Rockefeller Institute for Medical Research; and (as Cole states) in response to environmental pressures of all kinds, idealogic and intellectual. The "practical trial of full-time medicine" that Osler asked for in 1913 was delayed by the war but finally was provided 14 years later by a relatively inexperienced and brave doctor, aged 39, in Chicago.

WORLD WAR I

After McLean had returned from his 1916 trip to China and while he was engaged in the planning for the Peking Union Medical College, the United States entered World War I in April, 1917. McLean made a quick trip to China that summer to advance the local planning there and on his return to the United States was commissioned as a 1st Lieutenant in the U.S. Army Medical Corps. His first assignment—in December, 1917—was as a member of a mobile medical unit, of which the other two members were Robert L. Levy and Kenneth F. Maxcy. With the outbreak of an epidemic of measles, complicated by pneumonia and empyema, the unit was ordered to Camp Bowie, near Fort Worth, Texas, where deaths were occurring at the rate of 20 per day. The unit found itself engaged, not only in meeting the medical problems, but even more in reorganizing and retraining the medical officers assigned to the Camp Bowie hospital.

Such assignments had been made in a haphazard manner by the Office of the Surgeon General of the Army, and the result was a lack of skilled officers trained in internal medicine and able to meet emergencies. Theodore C. Janeway had been appointed chief of a Division of Internal Medicine in the Surgeon General's Office and had made a beginning at correcting this situation when he died, shortly after taking over this office. Warfield T. Longcope, also of the Johns Hopkins School of Medicine, was called to take his place, and he brought McLean to Washington to assist him. There McLean was engaged for some 6 months in improving the situation with respect to the recruiting and the assigning of personnel in internal medicine and in organizing the medical services of units being formed for duty in the American Expeditionary Forces. Here his experience at Camp Bowie, although it had lasted only 1 month, furnished an invaluable background of the needs of the hastily organized hospitals both in the United States and on foreign service.

McLean's services were requested by the Headquarters, Medical and Surgical Consultants, American Expeditionary Services, and he sailed in a convoy from New York on July 14, 1918. On arrival in Neufchateau, France, he was assigned to Brigadier General W. S. Thayer (professor of medicine, Johns Hopkins Medical School) as an aide, which meant as a personal confidant and general manager of his schedule of official and unofficial duties. Before the end of World War I this assignment was regularized by McLean's promotion to the rank of Major, and his designation as *Senior* Consultant in General Medicine, A.E.F. All through his life he has seemed to gravitate to areas of the world where there was important action in medicine. Harvey Cushing, writing in France on October 30, 1918, records the event of the day when McLean "brought a new novel and news" of the collapse of Mittel-Europa, the central nervous system of the German Empire.[5] (Fig. 1)

L. J. HENDERSON

There was the inevitable delay in returning to the United States after the Armistice, but on January 28, 1919, McLean was separated from the Army and was free to return to his China assignment. He visited Peking during the summer, where building was in progress, although delayed by the war. This left him with time free to return to the laboratory, and he spent the winter of 1919 to 1920 at Harvard University with



FIG. 2 Dr. McLean in his office at the new University of Chicago Clinics in 1927. William H. Welch appears in the etching on the wall behind him, as if for moral support. The clean desk reflects the efficiency of the young director of a progressive new medical school and hospital.

separate research laboratories, not made to conform to a pattern but to constitute an independent university discipline. He had the idea that he would pursue teaching and research as well as the administrative work of the office of professor of medicine. While the buildings were under construction, he spent one winter in Munich to broaden his experience by working with Friedrich von Mueller and another winter to resume his experiments with Van Slyke and his friends at the Rockefeller Institute.

In June, 1924, 220 members of the Rush Medical School Faculty and its student body became members of the University of Chicago. (However, the new hospital on the south side did not open until October, 1927.) The best of the old art and the new science of medicine began to work together at the University of Chicago before there were buildings to contain so much energy. The heat of the professional, academic, sociologic and economic fermentation of that era caused things to boil over. Alfred Cohn thought the unrest to be due to the fact that, in the minds of many in Chicago, the full-time plan was adopted only tentatively.[3]

From 1923 to 1932, young Professor McLean was immersed almost wholly in committee meetings, administrative problems and faculty feuds. A photograph of McLean taken during this period shows a portrait of Welch on the wall behind him as if there for moral support (Fig. 2). He expended almost all his energy on the drive to establish full-time medicine and had no time for research. A strong minority of the Rush faculty proposed that the University of Chicago abandon the full-time plan in favor of part-time private practice. Even if he were

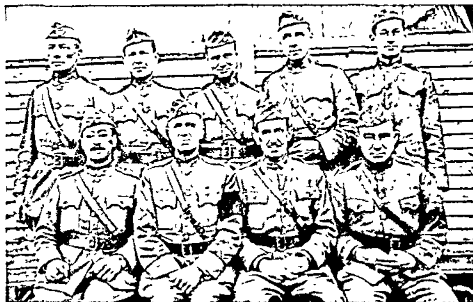


FIG. 1. Brigadier General William S. Thayer's Staff, American Expeditionary Force, Neufchateau, France, November, 1918. (*Standing, left to right*) Col. T. R. Boggs, Maj. F. W. Peabody, Maj. F. C. McLean, Lt. Col. G. B. Webb and Sgt. L. L. Derby. (*Seated, left to right*) Lt. Col. R. Dexter, Brig. Gen. W. S. Thayer, Col. W. T. Longcope and Lt. Col. A. E. Cohn.

L. J. Henderson. McLean was interested in the shift of chloride between red blood cells and plasma, and Henderson had just postulated the effect of oxygen upon the dissociation of hemoglobin as an acid. They joined forces, and, with the aid of Harry Murray, it was shown that the chloride shift was dependent on the variable acidity of hemoglobin. The story of this collaboration, and of McLean's part in it, is told in full in Henderson's monograph *Blood*.[10]

McLean has observed that Henderson's own contributions were mainly those of his mind, rather than of his laboratory, and that he was responsible for opening whole new areas for investigation. Recently McLean wrote that

> the privilege of working with him and observing him in action was responsible for giving me guidance and direction in my further activities [14]

THE CHINA CAMPAIGN

The physical structure of the Peking Union Medical College was completed in 1921. Having no opposition or competition from an organized medical hierarchy, McLean was able to establish, with little or no difficulty, high scientific standards and an excellent quality of correlated medical research, teaching and practice. When he returned to the University of Chicago to repeat this accomplishment, he found the task much more difficult in the United States than in China.

Before relating the history of the Chicago Campaign, it is important to note that, in Peking, McLean was not so burdened by administrative work as to be unable to contribute to one of the most important scientific advances to come out of Peking Union. Van Slyke was invited as a visiting professor during the winter of 1922 to 1923; during that time Van Slyke, Wu and McLean worked out the Gibbs-Donnan effect and a complete nomogram for (horse) blood. This work was a continuation of the studies begun with L. J. Henderson and assigned by him to McLean to continue with Van Slyke. McLean is said to consider this his own most important contribution to medical research. By others, his most significant contribution to American medicine is believed to be the implementation of the first complete full-time plan in an American university.

THE CHICAGO CAMPAIGN

In 1923, Franklin C. McLean planned the buildings and the organization of the new hospital and clinical departments of the University of Chicago. The architectural design reflected his advocacy of Rufus Cole's proposal that each clinical department should be free to develop in its own way in

academic hospital. The full-time program that was too expensive for Welch in 1907 suddenly became too expensive for McLean following the economic recession of 1929, but he had such great faith in the eventual success of full-time medicine that he appealed to the leaders of the Rush faculty for help. He proposed a solution, which was logical and had proved to be practical elsewhere in the United States at that time, that the doctors in private practice at the Presbyterian Hospital move into the new Billings Hospital and take over the hospital end of the operations in the same way that the New York Hospital now does at Cornell University and Presbyterian and Sloan Hospitals do at Columbia University Medical Center. An excerpt of the response to this invitation, signed by a committee of 9 senior members of the Rush faculty (approved by 91 members of the staff), was as follows:

> The University of Chicago admits that it is committed to "a reasonable trial in all good faith" of the Rockefeller idea in medical education. The present teaching on the south side is on that basis. Every man on this committee believes that the numerous failures of this plan indicate that it has already had a reasonable trial, and, in fact, has had more than a reasonable trial and that the Presbyterian Hospital and its staff would lose efficiency if obliged to function under such a plan.
>
> We would propose then one of two alternative conditions to meet this situation.
>
> 1. That the University conclude that it has already given a "reasonable trial" to the Rockefeller plan; that it change to an arrangement where no head of a department is a "full-time," thus leaving the administration and policies of the institution to those best informed as to the needs of medical care of patients and medical education. Since such part-time heads as would be appointed would not receive salaries, these funds would be available for the support of teaching and research by younger men.
>
> 2. That if the University does not wish to give up the experiment in full time at present, the Presbyterian Hospital be made one of two departments for the teaching of third and fourth year medical students. These departments would be coordinate but entirely independent of one another in administration and budget: One represented by the present full-time organization now on the south side, the other represented by the Presbyterian Hospital functioning as it does at the present time. (Undated, 1932.)

When McLean read these terms, he saw that they failed to include a source of financial support for full-time clinical teachers and immediately backed out of his proposal. Unfortunately, the Rush compromise appealed to the Board of Trustees of the University. McLean saw that he had to obtain additional funds to retain full-time men in teaching and research. Singlehanded he raised $500,000 (contributed by the Julius Rosenwald Fund, Albert Lasker and Max Epstein), and this acquisition eased the situation until the University Clinics got on their feet. Dallas B. Phemister appointed professor of surgery in 1925, supported McLean, and in 1949, in an address as retiring president of the American College of Surgeons, he concluded that

> under existing economic and social conditions in the United States, the most promising way of gradually placing education in clinical medicine on a uniform basis of organization and on an educational level that most nearly approximates the educational level of other university departments appears to be by the employment of full-time group-practice for the clinical departments of the medical school.

In 1933, McLean opponents—the group that had hoped to bury the full-time plan—succeeded in forcing at least his complete resignation in revenge for their defeat. It is ironic that the Rush faculty and the Rush organization that opposed McLean were later absorbed by the University of Illinois and are no longer in existence. It is significant that to Dallas Phemister, a product of the Rush faculty, McLean gives credit for much of the eventual success of the University of Chicago Clinics.

Dr. McLean was greatly disillusioned by the persistent attacks against his efforts to institute a full-time program. His career in

inclined to comply with this view, McLean was bound legally to retain contracts in force with the Rockefeller boards. In dealing with personality differences among the new and the old faculties, he encountered difficulties that an older and a more experienced clinician might have been able to foresee and avoid. He expressed opinions and took strong stands on issues that were of special interest to him; i.e., the medical care of Julius Rosenwald, the appointment of Franz Alexander in psychoanalysis, and the affiliation of the Provident Hospital. McLean's stand on controversial subjects, such as psychoanalysis, Negroes in medicine and medical care of a benefactor of the University, was based on strong convictions and was used against him with considerable effect.

In 1929, psychiatry was a controversial subject in Chicago, and McLean's efforts to support psychoanalysis led to criticism. A letter dated December 7, 1953, from Franz Alexander to Dr. Harry A. Wilmer contains an interesting account of the history of the introduction of Freud's work and the field of psychoanalysis in Chicago. In 1930, McLean recommended the appointment of Franz Alexander as visiting professor of psychoanalysis. When he gave his first lectures, one of the faculty members rose and declared that

> psychoanalysis is not based on experiments, it is not a science, and medical men should have no interest in this type of speculation.

Later, in the same letter, Alexander wrote as follows:

> During the whole year, Franklin McLean, keenly interested in my field, was my main adviser and he and his wife extended warm hospitality to me during my stay in Chicago. Dr. McLean, because of his consistent and courageous support of the cause of psychoanalysis in Chicago, himself got into serious difficulties with the University authorities

An excerpt of a memorandum that McLean wrote, with the advice and the assistance of Alexander, and circulated to those attending Dr Alexander's second lecture, is evidence of the personal interest that the young medical director had in introducing this new branch of medicine to the University of Chicago:

> It is a common phenomenon in the history of science that the stubborn and naive attempt to apply the methods of one science to the data of another different one, retards further development. The method of approach must be accurately adjusted to the specific nature of the material to be studied. Psychology *must employ psychological methods* and not the methods of physics or biology. The purpose of science is not to prove the efficiency of methods already discovered and tested, but to find adequate methods to investigate a specific field. Psychoanalysis has not the task of proving the efficiency of the methods of experimental sciences, but the efficiency of psychological methods.
>
> Two attempts to force the experimental methods of physics and physiology upon psychology have failed in recent times: (1) *experimental psychology* started by Fechner and Weber; and (2) *behaviorism.*
>
> The first one developed a science which supplies important data chiefly of physiological nature, which, however, have contributed very little to the understanding of problems of personality and behavior.
>
> Behaviorism similarly applied and developed several important discoveries in the physiology of reflexes, but excluding precisely psychological facts from the field of investigation, was able to contribute even less to real psychological knowledge.

Thirty years have elapsed since McLean made this statement, and time has not erased the strong opposition to psychoanalysis in many institutions in the United States. It is argued that in the grand strategy in medical science, planned, controlled experiments, or careful quantitative observation, has very general application. No one practices this policy more strictly than McLean in his own field, but he does not demand it of others working in another branch of medicine.

McLean's natural enemies, those who opposed the full-time plan, were able to dethrone him as soon as the Depression came; he became hard pressed to find the money to meet the high cost of operating an

clinical faculties in such a way as to afford fair compensation for the individual and to retain his main energies and time for his university position. These problems are being met in different ways in different institutions, and only time will determine the survival values of the various plans in effect. In the meantime, there is no shortage of personnel to fill available positions in the medical schools of America, and the University of Chicago, under its version of the full-time plan, is able to compete successfully with other institutions which, on the surface at least, may appear to offer more favorable terms to the members of their faculties.

The course of events in American medicine in the past 8 years, since this statement was written, has further increased the number of schools with full-time faculties. McLean was highly successful in introducing the newer scientific methods in medicine in Chicago, but as yet he has not been able to offer a solution of either the serious problem created by cycles of depression and inflation in the economics of modern medicine or the necessity of compensating scientists equally for teaching and for private practice. Perhaps there is no ready solution, but many of his friends, in both full-time and part-time practice or teaching all over the world, hope that McLean will share his experience with those who are now at work on this problem.

ORTHOPAEDIC SURGERY

McLean's contributions to orthopaedic surgery were both administrative and scientific; they began before he became preoccupied with the subject matter of the physiology of bone. In American medical schools only 30 years ago there were few laboratories in clinical departments, and the department of pathology was chiefly responsible for the research that was done in clinical subjects; there were very few full-time medical teachers or investigators. Even today there are many medical centers in the United States in which the division of orthopaedics has no research laboratories or budget of its own, notwithstanding the fact that it is responsible for more than 15 per cent of the total patient visits to all general hospitals. Orthopaedics has been retarded in its development in many institutions because of lack of full-time teachers, insufficient research workers, domination by the department of surgery and no representation in the academic senate.

This was not the state of affairs at the University of Chicago. During McLean's tenure of office, orthopaedics gained a favored position in the department of surgery and had the advantage of unusually ample clinical facilities.[19] Dr. Nathaniel Allison was appointed professor of surgery and chief of orthopaedics in 1931, and two buildings, designed for the care of crippled children, were assigned to him (The Gertrude Dunn Hicks Memorial and the Nancy Adele McElwee Memorial). For convalescent care of chronic bone diseases, such as tuberculosis and hematogenous osteomyelitis, the facilities of the Country Home for Convalescent Children, at Prince Crossing, Ill., were made available by a contract of affiliation with the University in 1927. Progress in medicine since 1927 has reduced the number of children with these crippling conditions in the United States and has suspended the need for such activities at the country sites, but out of this there developed a close relation of these convalescent homes and of their directing boards with the University.

When McLean turned his attention to the physiology of bone, as will be related below, he became interested also in the further progress of orthopaedics, and he has continued this interest, manifested in many ways, to the present day. He first participated in the Instructional Courses of the American Academy of Orthopaedic Surgeons in 1946, and the foreword to the present volume includes a significant quotation from his statement at the 1947 meeting. He has continued his participation in these courses to the present time. He was elected to membership in the Orthopaedic Research Society, an adjunct of the Academy, in 1957, and takes part in the annual meetings of this society.

McLean has always insisted that the nontraumatic diseases of the skeleton, including

the practice of medicine had received a mortal wound; he has not examined or treated a single patient since 1933. At the invitation of his old friend and teacher A. J. Carlson, he moved his office from the University Clinics to the Department of Physiology, having the consolation of responsibility for the structure of the full-time plan of research and practice that is still functioning so well and is being initiated in other medical schools in the United States.*

McLean's present views of the problems of the full-time system and trends in academic medicine were voiced in an address that he made at the twenty-fifth anniversary celebration of the University of Chicago Clinics in 1952.[11] He has never harbored ill feelings toward his associates or the University authorities but looks instead with pleasure and satisfaction on his past work. He offered the following objective observations for consideration by those who will have responsibility for *his* medical school in the future:

The early planners at the University of Chicago had in mind the conventional privately operated charity hospital and dispensary of the day. But before the Clinics were opened it had become apparent that the resources of the University were not sufficient to support a full-time staff and at the same time to operate a large charity hospital and dispensary. Under the pressure of need, subsequently made even more urgent by the depression of the thirties, there emerged the group practice plan. Perhaps the University has made a virtue of a necessity, but it is nevertheless true that the previously accepted viewpoint that clinical investigation and teaching are possible only with charity patients has been disproved. In the meantime, the patients who have received medical and surgical services have contributed largely to the cost of these services. To have performed these services and to have financed them as a charity for the needy sick, would have imposed an intolerable burden on the University.

This subject has broader implications than those which refer only to the financial operations of the University of Chicago Clinics. We hear on all sides of the enormous costs of medical education; of the need for additional financial support; of the requirement for large numbers of additional so-called "clinical beds"; of attempts to secure support to meet these needs from governmental or private sources, and of continuing failure to meet the mounting costs. As Dr. Phemister has pointed out, it is entirely possible that much of these costs, especially for professional services rendered, could be met by the patients, or by voluntary insurance plans, and the Chicago experience has pointed the way. There are many reasons for the failure of other institutions, already geared to the private practice of medicine, to adopt the Chicago plan, but to attempt even to recite these reasons would take us too far afield. It may be said, however, that the Chicago experience is available for study if and when there develops further interest in its applicability to other institutions.

That the University's version of the full-time plan has been financially an asset to the medical program is evident from what we have said. It is even more important that it has been successful in attracting and holding a distinguished faculty. It is clear that there are, as Dr. Cole predicted, many individuals of great ability and of the highest qualifications who are prepared to dedicate their lives and careers to accomplishment in teaching and research in medicine, even at considerable financial sacrifice to themselves. One need only attend one of the annual meetings of the national organizations devoted to clinical investigation to realize that there are, in the United States, literally thousands of young physicians, men and women, who desire more than anything else to participate in the great advance in scientific medicine. The University of Chicago recruits its medical faculties from among such individuals. There are still problems, at the University of Chicago and elsewhere, with reference to adjustment of the earning capacities of the

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* Prof. Friedrich von Muller, with whom McLean had worked "actively" in Munich, recorded the impression that a visiting clinician would receive from the developments in medicine in Chicago in 1933:

"He (McLean) guided me tirelessly through the enormous buildings of the University of Chicago Clinics, which had been built by the Rockefeller Foundation But here I must point out that this new establishment was much more concerned with experimental investigations on mice, rats and monkeys than on sick people. It turned out shortly thereafter that the Rockefeller money was inadequate to fill the expensive building with substantial medical activity, and McLean gave up his post." (von Muller, F.· Lebenserinnerungen, p. 229, Munich, J. F. Lehmanns Verlag, 1951)

ical warfare agents, Franklin C. McLean was selected as director of the project. This laboratory, at first under the cover name *Respiration Laboratory*, was housed in temporary buildings in a vacant lot across the street from McLean's laboratory in the physiology building of the University and screened a large number of candidate agents. Among other achievements, the laboratory discovered the potentiality of the nitrogen mustards as chemotherapeutic agents, and this eventually had practical application in the treatment of leukemia and other malignant diseases.

From 1943 to 1945, Dr. McLean served as Lieutenant Colonel, Medical Corps, United States Army, assigned to the Medical Division of the Chemical Warfare Service. During this period he also served as director of toxicology at Edgewood Arsenal, Maryland; as chief of the Persistent Section San Jose Project, Panama; as a member of the British-American Coordination Staff; and as deputy chief of the Medical Division. For his contributions to the Chemical Warfare Service, including those in Chicago and in the Army, he was awarded the Legion of Merit in 1945, the Army Commendation Ribbon in 1947, and the Army and Navy Departments' Certificate of Appreciation in 1947. His citation for the Legion of Merit reads in part:

> Lieutenant Colonel McLean was a key factor in evaluating the biological aspects of all research and testing of gas warfare equipment performed by the United States and the British Commonwealth. In charge of research and development, he directed the entire scientific effort of the Medical Division. His unsparing use of unusual professional talents and abilities reflects great credit and distinction upon himself and the Military Service.



FIG. 4. Lieutenant Colonel McLean in 1944, following assignment to the Medical Division of the Chemical Warfare Service.

Following the war McLean retired from the Army with the rank of Colonel and returned to the University of Chicago. However, he was soon called upon for further service, this time on behalf of the Atomic Energy Commission, with responsibilities at Los Alamos, as noted below, at the University of Chicago and in Washington. At Chicago he became director of a special A.E.C. project, operated in collaboration with the reorganized Toxicity Laboratory directed by Julius Coon. During this period, Colonel John R. Hall, MC, USA, was assigned as associate to McLean, and he enjoyed the experience so thoroughly that he almost regretted being recalled to Washington. McLean, in turn, thought himself blessed when the Army sent Colonel Hall to him. In a letter to the Surgeon General, dated August 7, 1955, he wrote:

> I wish to go beyond the technicalities of this matter (Colonel Hall's papers for upgrading), and to say that in all my contacts with

sity of London, who had demonstrated alkaline phosphatase activity in bone and proposed a theory of its role in the physiology of calcification. McLean found it difficult to believe that alkaline phosphatase was a critical factor because (1) the optimum pH of the enzyme is high, namely, 9.4; (2) the concentration of phosphoric esters in the tissue fluids is too low; and (3) other tissues, which do not calcify, are rich in an identical or a similar enzyme. He set out to educate himself about bone as a tissue. McLean consulted Smith Freeman and collaborated with him on blood changes in experimental rickets. He joined Lillian Eichelberger in an investigation of the distribution of calcium and magnesium between the cells and the extracellular fluids of skeletal muscle. He also consulted William Bloom, who had completed and edited Maximow's *Textbook of Histology* and who encouraged him to renew his study of anatomy. The result was a collaboration that has lasted more than 20 years, has produced many important papers on histophysiology of bone and has included contributions by McLean to successive revisions of the chapter on bone in Maximow and Bloom's *Textbook of Histology*. The lively discussions that occurred while McLean was learning about connective tissue from Bloom were witnessed with considerable delight by R. H. McCoy, Esther DaCosta, Evelyn Smith Ross, Marie Hinrichs and others, who assisted in experiments going on in McLean's laboratory.

A product of this association was a new understanding of the relationship between calcification and ossification and the effect of hormones on these processes in growing bone. During the summers, both McLean and Bloom moved to Randolph Center, Vt., and lived on adjoining farms. For the 2 summer months of one of these years a medical student from Johns Hopkins (the author of this article) was placed in charge of the laboratory and given a free hand to follow wherever the research might lead.

McLean rarely indulged in speculation and taught that an experiment should be set up on an orderly pattern; yet he always gave his support to any scientist who had the urge to work on an idea all his own. Bernard Ross, a medical student, fascinated by parasitology, was captivated by the idea that he should study calcification of trichina cysts in vitro;[17] an accident happened when the spray from the neck of a syringe struck his eye and he developed trichinosis as the result of the passage of larvae through the tear duct into the oral cavity.

Margaret and William Bloom called McLean's attention to bone metabolism in birds, and this subject, as well as the turnover of calcium in the laying hen, the estrogenized mouse and the estrogenized rat, still occupies his mind. He also watches the literature closely on the mechanism of calcification in vitro and has corresponded with William and Margaret Neuman, of the University of Rochester, Harold Copp, of the University of Vancouver, and Melvin Glimcher, of Massachusetts Institute of Technology, and others on this and related problems. The flexibility of McLean's intellect is most extraordinary, and this perhaps is his most important quality as a scientist. He was ready to propose a different approach to the problem of calcification at the Lankenau Hospital Research Conference on October 30, 1958, when he said:

> There is now reason to believe that phosphate, heretofore regarded as the passive member of the calcium-phosphorus team in calcification, may be found to provide such activity or energy as may be necessary for this process or more particularly for its initiation or seeding. In this case, the passive or secondary role may have to be assigned to calcium.

CHEMICAL WARFARE, RADIATION BIOLOGY AND WORLD WAR II

Before the entry of the United States into World War II, when the National Defense Research Committee hastened to organize a research laboratory at the University of Chicago in order to study prospective chem-

this resulted in termination of the Los Alamos Campaign.

PHYSIOLOGY OF BONE AND THE MACY CONFERENCES

The course of his research on the state of calcium in the fluids of the body led Dr. McLean into pathways covering every aspect of the biochemistry and the physiology of bone. An important source of stimulation to him, between 1946 and 1953, was Frank Fremont-Smith and the Macy Conference Program on Metabolic Aspects of Convalescence and on Metabolic Interrelations. Dr. McLean's published remarks at these conferences were relatively few and rarely were given without a direct request from the chairman, but they were always charged with meaning and pertinent questions bearing precisely on the data under consideration at the time.

Dr. Franklin McLean carried on a friendly but spirited argument with Dr. Fuller Albright on hyperparathyroidism for 15 years. Albright contended that the phosphaturic effect of parathyroid hormone was its chief mode of action, while McLean argued that the effect on bone resorption was not dependent on phosphaturia. Albright, a brilliant scientist and logician, presented a mass of excellent clinical data that is still without flaw, and clearly he had the last word for the 10 years between 1937 and 1947. In 1948, Barnicot demonstrated the local action of parathyroid hormone in experimental animals; this corroborated McLean's 1937 report, with Bloom as coauthor, in *Science*, in which they described direct effects upon the bone cells; dedifferentiation of osteocytes and osteoblasts into osteoclasts and resorption of bone within hours after injection of parathyroid hormone. The kidney is still to be regarded a target, and Albright's data continue to gather new support every day. It is interesting to speculate on how the Albright-McLean debate about the primary action of parathyroid hormone would stand today had Fuller Albright not been withdrawn from the race by an unfortunate complication of parkinsonism.

Recently McLean was challenged by one of his students to define basic research, and he replied:

> It is easier to define applied research than basic research. When a company spends a lot of money trying to find a new drug they can exploit, that is clearly applied research.

One pharmaceutical concern consulted him after they made a search for an estrogen with a specific effect on bone but with little or none on the uterus. A friend asked him for an opinion on the project, and he wrote:

> They spent a lot of money on this attempt, when some attention to fundamentals of the effects of estrogens on bone in mammals might have stopped them at the beginning, instead of later, when they abandoned the project. If they would go into the fundamentals without trying to find something they can sell, that can be basic research. And a little basic research might easily save them thousands of dollars in following false leads. I suppose they are too eager to jump the gun in fear that someone may beat them to the advertising columns of the J.A.M.A.

In Rufus Cole's letter to McLean, dated July 20, 1959, he said that

> men in the hospital [Rockefeller Institute]* might work in any problem, no matter how far removed from practical applications, but . . . in the backs of their minds, there should be the desire to learn more about disease as it occurs in man in the hope of relieving men from its effects. . . . It is possible that the same amount of thought and energy employed in pure research might have yielded even greater returns. No one knows.

McLean integrated research and applied science by demonstrating that the study of disease in animals aids in reaching a clearer understanding of disease in man and that the two are inseparable fields of endeavor. Later, Franklin McLean gradually went even farther, and now he supports Alfred Cohn's view[2] that research work should be a field of endeavor in its own right with no foregoing obligation toward practical application. While he had, and today still has, many famous and authoritative opponents on this score, his own work proves that applied science lives by

* Author's addition.

this officer, and they have been many over a considerable period of time, I have found him to be a superior representative of the Medical Corps in all respects both professional and personal

This sample of McLean's letter-writing reflects the pleasure that he finds in contributing to the advancement of his associates. Also associated with McLean during this period was John H. Rust, D.V.M., Ph.D., until recently Colonel in the Veterinary Corps, United States Army. He collaborated on various aspects of whole-body irradiation and recently was appointed professor of pharmacology and head of the Section of Nuclear Medicine at the University of Chicago.

In more recent years the Toxicity Laboratory has continued in the field of radiation biology, now as the Air Force Radiation Laboratory under the direction of Kenneth P. DuBois. Thus the laboratory, established as an emergency facility under McLean's direction in 1941, is still highly productive, and it has made many contributions to fundamental biology, as well as to the needs of the Armed Forces.

THE LOS ALAMOS CAMPAIGN

In 1946, Dr. McLean resumed a schedule of lectures in physiology and was soon surrounded by staff, fellows and graduate students—Dr. Henry Hemin, Zelma Baker Miller, Magdalene S Carttar, Dr. Jerome Waldman, Ann M. Budy, M. Heller and others. Some had been in military service, had just returned to civilian life and were interested in research work on calcium and bone metabolism under his direction. Between lectures and conferences with students, he made frequent trips to New Mexico, where, in the capacity of consultant to the Santa Fe Operations Office, Los Alamos, he was planning the construction of medical research laboratories and a hospital for the Atomic Energy Commission. As he had done previously in China and at the University of Chicago Clinics, he attempted to consolidate research (here on radiation biology) and medical service to the workers and their families. It would seem that because of the scant knowledge of nuclear medicine and the health hazards, known as well as unknown at that time, this would be an ideal place for McLean's policy of housing, under one roof, active research and practice of medicine. However, he failed to persuade the administrative chiefs of the practical value of this measure. The research laboratories were being operated by the University of California. The Atomic Energy Commission, without coming to a previous agreement with the scientists of the University of California, asked him to assume the direction of the medical services for the community of Los Alamos, and he found himself immersed in a complicated situation.

Some weeks before he was to attend a special conference at Los Alamos to propose a plan and working agreement, he was run over near the campus of the University of Chicago by a hot-rod automobile and sustained a head injury. At Los Alamos, he presented a comprehensive community medical program while suffering from what soon afterward was diagnosed as a subdural hematoma' From there he traveled to Los Angeles to visit a former pupil working at the University of California. His pupil made an examination and read the chapter to him on subdural hematoma in *Neurology*, a textbook written by Dr. McLean's good friend Dr. Roy Grinker. It was found that one of the most prominent symptoms of interference with his cerebral function was his anxiety about time. Normally, Dr. McLean never seemed to lose track of time. It was his habit to arrive at the airport at the exact time to check in for boarding. After his head injury, he was ill at ease and continuously unhappy about the time of day, and on one occasion he insited on arriving at the airport 1 hour early! He suddenly became ataxic and disoriented and was hospitalized, fortunately in time, by Dr. Grinker for a craniotomy by Dr. Paul Bucy. The hematoma was removed under local anesthesia, and the patient was relieved immediately and permanently of his symptoms. However,

ability to write; he is a keen proofreader and his book on bone has been said to be perfect grammatically and typographically.

In 1957, Dr. McLean was awarded the degree of M.D., *honoris causa*, by the University of Lund, in Sweden, for his work on the parathyroid glands and on the physiology of the skeletal system. This is the same university from which A. J. Carlson and Otto Folin, both of Swedish birth, received the same degree in 1919, on the occasion of the 250th anniversary of the founding of the university. So far as can be learned, Franklin McLean is the first native-born American to receive this honor. His honorary membership in the American Academy of Orthopaedic Surgeons, conferred in 1959, has been referred to above. In this volume, his pupils, friends and associates take pleasure in presenting him with best birthday wishes for further success and a new collection of papers on bone that it is hoped will be useful to him in his work and to the readers of *Clinical Orthopaedics* (Fig. 5).



Fig. 5. Dr. McLean in 1957 following the award of the degree of M.D., *honoris causa*. He is wearing the traditional cap and dress for the commencement ceremony at the University of Lund (Sweden). This is the same institution from which his first teacher, A. J. Carlson, received the same degree in 1919, on the occasion of the 250th anniversary of the founding of the university.

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research and that research contributes most if allowed to progress untethered. McLean's bibliography contains such important and practical documents as (1) the variable acidity of hemoglobin in the transport of oxygen in the blood; (2) one of the earliest mathematic formulations for measuring urea clearance; (3) the McLean-Hastings nomogram for measuring calcium ion concentration of the blood; (4) the rationale of the vitamin D treatment of hypoparathyroidism; (5) the most generally accepted explanation of the relationship between calcification and ossification in bone growth and repair; and (6) the feedback theory of the mode of action of the parathyroid hormone.

CURRENT ACTIVITIES

Since 1954, the annual Gordon Research Conferences on the Chemistry, Physiology and Structure of Bones and Teeth have been held near McLean's summer home in Vermont—at Meriden, N. H. Every year Dr. McLean and his charming wife Helen invite all the conferees to visit their home. Lively arguments about calcium and bone metabolism go on all afternoon and continue on the road back to New Hampshire and far into the night.

Occasionally Dr. McLean accompanies his wife, Dr. Helen Vincent McLean, a practicing psychiatrist and psychoanalyst, to meetings of psychiatric societies. The Group for the Advancement of Psychiatry invited *him* to read a paper on November 9, 1958! His subject was "Psychological Aspects of the Nuclear Arms Race," and he observed that the basis of the cold war between Russia and the United States was fear and aggression.

At the present time Franklin McLean serves on the Technical Advisory Panel on Biological and Chemical Warfare under appointment by the Department of Defense and hastens to respond to any request from the Armed Forces for his advice However, for the most part he is at the University working on a research project with Dr. Ann M. Budy on radioactive estrogen, with Dr. Isador Gersh and Dr. Zelma Molnar on electron microscopy of bone, and with Dr. John L. Howard on osteoporosis of the human spine. McLean is also collaborating with John Marshall and Robert Rowland, who are working at the Argonne National Laboratories on the application of methods of biophysics to problems of bone metabolism. He continues to support the policy of integration of medical education, research and practice. At a recent conference at the Institute of Medicine of Chicago, of which he held the office of president during 1959, he favored the view that

> responsibility for conducting research and for selecting areas requiring additional study should rest largely with research centers in medical schools and hospitals. The development of specialized centers for study of chronic diseases, apart from the established centers for research in general medicine and surgery, is not practicable, and should not be encouraged [13]

McLean's articles are attractive to students because they include discriminating reviews of the literature. Twenty-six years ago, when he began to "re-educate" himself, McLean designed a cross-filing system of the literature on all aspects of the physiology of bone. Under continuous cultivation by Dr. McLean and Dr. Ann Budy, the card and reprint file is up to date and is large enough to fill a room. In 1955, when the Reference Division of the Armed Forces Medical Library (the largest institution of its kind in the United States) undertook to make a bibliography on "The Structure, Composition, and Growth of Bone, 1930 to 1953," he was asked to pack up his reference cards and send them to Washington to help accomplish this arduous task. Estelle Brodman wrote the following acknowledgment in the introduction to this document on March 1, 1955:

> Dr. McLean further cooperated by generously placing at our disposal his extensive personal file, collected over the past twenty years, thus making possible the checking of our listing against his for additions not found through the usual indexing and abstracting services

McLean's advice is often sought by editors for both his grasp of scientific matters and

the picture further. Recently, these questions have been clarified mainly by the work of Carlström and collaborators and Trautz. Carlström *et al.* examined specimens of natural hydroxyapatites both chemically and crystallographically. They found that the carbonate content varied from a fraction of 1 per cent to several per cent in specimens from different localities. Furthermore, they found no correlation between the lengths of the crystallographic axes and the carbonate content. The only correlation that eventually could be traced was possibly the dimensions of the lattice parameters with the amount of fluorine or positive ions, such as magnesium. The fluorine ion is known to substitute in the hydroxyapatite lattice and the end member; fluorapatite is the only one of these compounds that has been subjected to a complete crystallographic analysis. Combined polarized light and electron microscopy studies seemed to indicate that the natural mineral apatites containing the highest amounts of carbonate consisted of an amorphous matrix with crystalline areas within it. Most probably the amorphous matrix consisted of calcium carbonate, whereas the crystalline areas were hydroxyapatite where the hydroxyl was more or less substituted by fluorine. Thus, even in mineralogic specimens, it appeared that a compound of the type carbonate-hydroxyapatite did not exist. The mineral apatite is a two-phase system of apatite and carbonate, and the latter is in an amorphous state; therefore, it cannot give any diffraction lines. This finding removes one of the main arguments regarding the nature of the bone apatite, McConnell, among others, claiming the existence of the carbonate apatite on the basis of his investigations of the mineralogic hydroxyapatites. Thus it now appears safe to accept the idea that the main mineral phase in the bone mineral salt is hydroxyapatite with amorphous calcium carbonate absorbed into it in some way or another.

Another question—a puzzling one—is, How large are the crystalline complexes in bone tissue? That is, What is the size of the crystallites? From studies in the polarizing microscope, W. J. Schmidt, in the early twenties, deduced that the mineral phase existed as small thin units, and he expressed the view that these were adsorbed in an oriented way on the collagen fibers.

There are two methods of studying the size of the bone crystallites: the direct one is to try to observe them in the electron microscope; the indirect way is to study the actual profiles of the x-ray diffraction lines. The latter method is possible due to the development of high precision goniometers, so that the profile can be obtained with a high degree of certainty. This makes it possible to apply mathematical principles to the interpretation of the distribution of the intensity of the scattered x-rays in the profile. Thin sectioning—for example, with a diamond knife—permits the production of sections of bone tissue thin enough to be studied in the electron microscope. The findings of the electron microscopic work vary, but there are some general common findings. The crystallites seem to be needle shaped and elongated, the width varies between 30 and 60 A, the length varies from a few hundred to a thousand A or more, depending on specimen preparation. Until recently, the study of the width and the shape of the x-ray diffraction profiles could be made only on a few lines, and especially on the 002-line. Taking the integral half width of this line and ascribing the broadening of the line only to the size factor, one obtains a value of the length of the particles of 220 A. However, measuring the profile of only one single line can give erroneous results, as there are other factors besides size—strain, for example—that may cause a line broadening. In order to tackle these problems, Dr. Carlström and Dr. Glas, at the Department of Medical Physics, Karolinska Institutet, prepared oriented specimens by which they could measure the profiles of lines also of higher orders and thereby distinguish between strain and crys-

4

The Structure of Bone; an Excursion into Molecular Biology

ARNE ENGSTRÖM*

The study of the structure and the function of the mineralized tissues presents an interesting example of scientific progress. Omitting the classic descriptions of the shape and its relation to the functioning of the skeletal system, one may say that around the turn of the century there was a fairly lively interest in scientific contributions dealing with these tissues. It was the era of microscopic anatomy and contributions, such as those of Gebhardt and Petersen, who described the microscopic anatomy of bone tissue. Their works are outstanding, judged even by today's standards. Later on, interest in the composition, the building and the functioning of the skeleton had become practically nil, only a few researchers continuing to devote their energies to mineralized tissues This thorough and persistent work of a few scientists is of value today when one realizes the great importance of the skeleton as involved in the homeostatic mechanism of the organism. Furthermore, the existence of bone-seeking radioisotopes in the fallout after atomic bomb tests increases still more the scientific interest in mineralized tissues. A group that has studied mineralized tissues continuously is that headed by Prof. Franklin McLean. His numerous contributions on a wide range of problems pertinent to bone structure and bone physiology have contributed substantially to our understanding His keen interest in these and related problems is illustrated by the fact that when the present author's first scientific contributions were published in Sweden during the last years of World War II, the first letters to arrive from abroad asking for information about the work were from Prof. McLean.

Against this background, I propose to give a brief account of certain aspects of bone molecular structure and physiology. Since the early work of de Jong, in 1926, x-ray diffraction technics have been applied to the study of the molecular structure of bone. Originally de Jong deduced that the mineral phase in bone closely resembled that of the mineral hydroxyapatite. Also, he could show that the crystallites were small and elongated and that the crystallographic c-axis seemed to be aligned after the long direction of the long bones. Since that first classic work, numerous attempts have been made to pry into the nature of bone salt. Many ideas have been advanced, especially as regards the role of the carbonate in mineralized tissues. For a long time there were strong arguments, led on the one hand by McConnell and on the other by Hendricks and others regarding the position of the carbonate in the apatite lattice. McConnell claimed that the bone salt was a carbonate apatite, whereas the others maintained that it was a pure hydroxyapatite, the carbonate being a separate phase. Also, the introduction of the concept "α-tricalciumphosphate," by Dallemagne and co-workers, complicated

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the future, the field of the molecular biology of mineralized tissues most probably will provide an exciting and a rewarding research field, and the system of organic-inorganic matter, although extremely complicated, is well suited for the application of modern biophysical methods.

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Le Structura del Ossos: un Excurso a in le Biologia Molecular

Summario in Interlingua

Es revistate le ideas que on ha disvelopпate con respecto al organisation molecular del componentes inorganic in le ossos e dentes. Recente experimentos roentgenocrystallographic e studios electronomicroscopic tende a indicar que le crystallites matur del ossos ha un longor de circa 600 A e un largor de circa 40 A e que lor conformation structural es plus o minus distorquite. Le soliditate de iste constatationes—e de alteres concernente le email dental—es discutite criticamente.

tallite size. They prepared specimens by aligning in a stack several hundred thin fish bones, which from earlier experiments were known to be well oriented, and embedding this packet in plastic. From this pile were prepared longitudinal and transversal sections having an optimal thickness for x-ray diffraction studies. By taking the width of the lines belonging to the 002-group (higher orders), they showed that the length of the crystallites were about 600 A and that they had a somewhat kinked structure or possibly were deformed in other ways. According to the same type of investigation, the width of the particles is about 40 to 50 A. Thus, these results of x-ray diffraction studies indicate that in mature bone the particles are about 600 A long and about $\frac{1}{10}$ or less of that value in diameter. These investigations agree relatively well with investigations in the electron microscope. Similar studies on enamel indicate that the crystallites in enamel have a length of about 1,500 A and a width of about 600 A. Naturally, the values of the dimensions of the crystallites deduced from x-ray diffraction data are a kind of mean values. Unfortunately, due to the nature of the x-ray scattering process, the very smallest particles give practically no lines at all; their scattering effect is a general increase of the background Hence we are not able to derive a distributional curve of particle sizes, which would be the only correct way to express the crystallite size in bone and in teeth. At the moment we cannot say if the "mean" value derived from the x-ray diffraction procedure is the median value or is shifted toward larger particle sizes in the distribution curve, which undoubtedly exists. However, as mentioned above, both x-ray and electron microscopic data already show that the width of the elongated particles is only 3 to 4 unit cells. Therefore, this smallness of "core" provides the great surface area and the variable pattern of surface changes giving the characteristic reactivity of the bone salt.

Recently there have been some investigations on the structure of the Sr-Ca-apatites to find out whether the Sr ion substitutes for the Ca ion in the crystallographic lattice or whether Sr becomes adsorbed on the surface of the small calcium apatite crystallites and eventually fixed by epitaxy. In trying to coprecipitate Sr and Ca apatites, Lagergren and Carlström found that not very large quantities of Sr entered the lattice itself, and they concluded that a large amount of Sr in the wet procedure was adsorbed on the surface. Naturally, heating the mixed apatites produces compounds with unit cell dimensions corresponding to the Sr to Ca ratio Recently, however, other laboratories found that Sr under optimal conditions in the wet precipitation could enter the lattice. Whether or not this takes place in the living system is doubtful, as Glas reports that in animals that had been kept on a high Sr diet and holding as much as 10 per cent Sr in their bones, there was no measurable shift of unit cell dimensions in comparison with calcium hydroxyapatite. In order to obtain a clear picture of the crystallographic conditions of the Sr-Ca-apatites, more work has to be done, and this is in progress.

In summary, our present knowledge of the size and the molecular structure of the inorganic component in bone and in teeth is fairly well established today. However, there are a great number of points in the molecular biology of bone that still require clarification. Is there a specific bond between the collagen and the apatites? Experiments of the type pursued by Neuman and by Glimcher may give an answer to this question The first steps of deposition of minerals in structures, later to become bone, are known only in the very broadest outlines Perhaps further refined electron microscopy will shed light on the matter. The peculiar surface characteristics of the small apatite particles, which determine the reactivity pattern of bone tissue, have to be studied more in detail, and Neuman's group already has produced highly interesting results on the chemical dynamics of the apatites. Thus, in

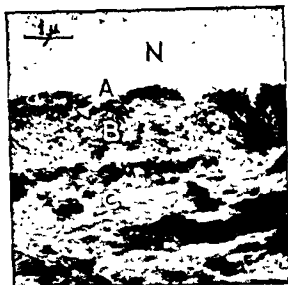


FIG. 1. Electron micrograph of an unstained coronal section of parietal bone of a 1-week-old mouse. The section was prepared by freezing and drying with postfixation in 95 per cent alcohol. A portion of an osteoblast with nucleus (N) is at the top. Adjacent to this is a pale region, the uncalcified osteoid (A). Next is the region of noncrystalline mineral (B). Below this layer is the dense crystalline bone (C). Cross bandings in the collagen are barely visible. Bar = 1 μ. × 11,000.



FIG. 2. Electron micrograph of a section of parietal bone prepared and oriented in the same manner as in Figure 1. Layer of amorphous bone mineral (Zone B in Figure 1) showing the arrangement of dense circular or oval figures. In some areas they appear to form chains. The zone of transition to the crystalline layer is at the lower portion of the illustration. Bar = 0.1 μ. Approx. × 68,000.

an attempt to account for these differences.

The parietal bone was selected for study of bone crystal dimensions because (1) ossification occurs directly in connective tissue without the influence of cartilage, and (2) it is thin and can be prepared without ice crystal formation. The bones of 1- to 2-week-old mice were fixed by freezing and drying. The specimens were oriented coronally, sagittally or transversely to the long axis of the body. Essentially the same structures appear in the different orientations. Sections of unstained, undecalcified specimens were viewed in a Philips EM 100A electron microscope and the Siemens Elmiskop I. Electron micrographs were made on Adox KB-14 film or Gevaert contrast plates.

An electron micrograph of an unstained coronal section of a 1-week-old mouse is shown in Figure 1. The section is from a region lateral to the sagittal suture. In the area between the osteoblast and the dense layer is a pale zone, the uncalcified osteoid. This region contains vacuoles of about 500 to 1,000 A. The vacuoles are not shown in Figure 1. They are visible at higher magnifications and when electron micrographs are exposed to show the organic material. Fibrillar organization cannot be detected in this zone of uncalcified osteoid either in unstained or stained preparations. However, in stained preparations, collagen fibrils can be detected on the periosteal side filling the space between the osteoblasts and the periosteal cells.

Between the uncalcified osteoid and the crystal-rich zone of bone a layer is visible

5

Additional Observations on Bone Crystal Dimensions*

ZELMA MOLNAR, M.D.

The study of dimensions of the crystals of bone mineral has stimulated investigators from many disciplines over a long period of time. However, it has been only within the past decade that our understanding of the dimensions has been advanced by the visualization of bone crystals with the use of the electron microscope. The historical background of the problems confronting investigators and of their progress with the availability of new technics was presented in detail in a review by Neuman and Neuman in 1953.[5] Robinson and Bishop[8] were the first to publish a method whereby bone crystals could be visualized; subsequently the definitive study of the dimensions of bone crystals and their association with collagen fibrils was reported in a series of papers.[6,7] This intensive study aroused considerable interest, and many investigators attempted more precise measurements of the bone mineral. Prior to this first paper on methodology, Ascenzi[1] reported in 1949 that microcrystals were not recognizable with a magnification of 18,000 diameters. In the same year, Wolpers[13] published an electron micrograph of bone showing densely packed needle-shaped crystals, 30 to 60 A in width and 400 to 1,000 A in length.

With new developments in electron microscopy and modifications of technics, other dimensions and shapes of bone crystals were reported. Robinson[6,7] described bone crystals with average dimensions of about 500 × 250 × 100 A. Variations in shape were reported as tabular plaques or platelike and needle-shaped particles, depending upon the orientation and the preparation of fragmented bone. In the same year, Robinson and Watson[10] described crystals as tabular-shaped plates or plaques in sections of undecalcified bone with dimensions of 25 to 50 A in thickness and 350 to 400 A in length and width. In 1956, Scott and Pease[11] reported dimensions of crystals of kitten bone as 50 × 400 to 500 A. Later, Robinson and Cameron[9] described single crystals measuring 25 to 75 A in thickness and 25 to 750 A in length. Fernández-Morán and Engström[3] reported needle-shaped crystallites 40 to 75 A in width and 200 A in length, with a periodicity of 50 to 60 A. In the same year, Speckman and Norris[12] reported dimensions of bone crystallites as 50 A thick and usually 600 to 700 A in length. Durning[2] described bone crystals as 40 to 60 A in width and 140 to 300 A in length. Recently, the writer of this chapter[4] reported bone crystals as 30 to 50 A in width, with an average length of 175 A.

There appears to be good agreement that the width of crystals ranges from 25 to 75 A, with an average of 50 A. The discrepancy arises in the dimensions in length. The results herein reported are those found in

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over several major periods of the collagen fibrils; some were traced up to a length of 3,000 to 4,000 A. All rods exhibited subunits of about 50 A particles. An interesting observation was the finding of marked differences in densities within the rods (Fig. 3). Electron diffractographs of such fields reveal the rods as *crystals*. Occasionally there is an arcing of the 002 ring, indicating that, in some areas, the crystals with their c-axes are parallel to the long axis of the collagen fibril.

SUMMARY AND CONCLUSIONS

The differences in the dimensions in length of a bone crystal observed in a given electron micrograph and reported in the literature, as well as those presented in this study, can be explained as follows:

1. The deposition of microcrystals as particles of about 30 × 50 A follows a pattern within the major period of the collagen fibril; the particles are deposited side by side across the subbands of the fibril.

2. Later, more such microcrystals are deposited between these subbands, thereby establishing an end-to-end relationship with the pre-existing microcrystals in this fibril. When this takes place, the periodicity of the collagen fibril is obscured.

3. The direction of elongation of a given crystal is influenced by the presence of microcrystals in the same or neighboring fibrils; i.e., the crystal will deflect in the direction of the closest microcrystal and will establish an end-to-end relationship in this new plane and direction.

4. Owing to these irregular deflections, only a part of the crystal can be observed, even in sections oriented parallel to the direction of the collagen fibril.

The dimensions of the crystals per se in different species probably do not differ much in ultrastructural appearance. There is good agreement that the crystals have a well-defined width, ranging from 25 to 75 A, with an average of 50 A. Based on the findings herein reported, it can be postulated that the crystals are composed of chains of microcrystals in an end-to-end relationship from an original length of about 50 A up to an unlimited length. The growth of crystals may be influenced by the remodeling process; more space is available through bone resorption for new crystal deposition.

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where fibrils and bone salts appear simultaneously. In this region circles or ovals ranging from 60 to 200 A in diameter can be seen forming chainlike structures, as if outlining fibrils; these structures have dense walls of about 40 A in thickness. A higher magnification of this area is illustrated in Figure 2. The dense component is not resolvable further. Electron diffractographs of this region show a hazy ring, suggesting that the dense material is composed either of amorphous bone salts or of crystals smaller than the resolution of the electron microscope.

The collagen fibrils in the crystal-rich part of the bone are oriented and aligned more or less parallel to the surface of the bone. In some areas the fibrils show darker and lighter bands with a periodicity of 510 to 550 A. These bands can be resolved into subbands, across which small dense particles (microcrystals), 30 × 50 A, are arranged side by side. In near-by areas more such particles are deposited between these subbands, thereby establishing an end-to-end relationship with the pre-existing particles *along the long axis of the collagen fibril.* In this manner, rods are formed extending over the lighter bands; they are numerous and so long that they obscure the periodicity of the fibrils. Later, rods are deposited between the fibrils, thus obscuring the fibrillar pattern, leaving space only for the lacunae and the canaliculi of the osteocytes and their processes. The length of the rods vary markedly from about 50 to 1,000 A. The rods were observed to curve and change both in plane and in direction, frequently extending

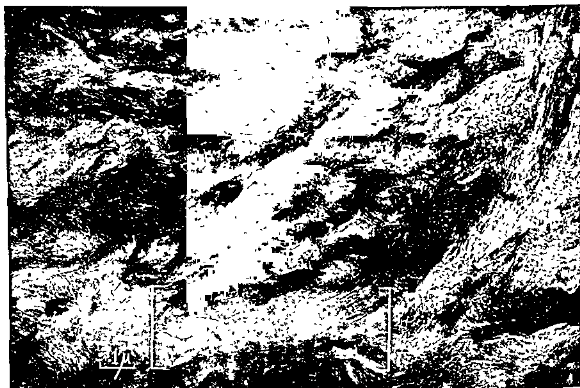


FIG 3. Electron micrograph of a section of parietal bone cut transversely to the long axis of the body. The section was prepared in the same manner as in Figure 1. This is the region of bone rich in crystals (Zone C in Figure 1). The area enclosed in brackets is a part of a collagen fibril with dense and less dense bands. Within each band are smaller subbands; each consists of dense particles with an average size of 30 × 50 A. They are aligned side by side across the fibril. The long axis of the fibril is horizontal at this site. In adjacent fibrils, the crystals are longer and are not arranged with any notable periodicity; the subunits are distinguishable within the crystals. Bar = 0 1 μ. × 76,000.

6

The Ivory Core of Tusks and Teeth*

REIDAR F. SOGNNAES, D.M.D., PH.D.†

The term *ivory* connotes many things other than what in fact it is: a form of dentin, the bone hard core of the dental organs in higher animals. This exquisite biologic substance has fascinated the human mind since the beginning of cultural evolution: the distant antiquity, when, in fumbling baby fashion, the grasping of objects and ideas went hand in hand; when Homo sapiens first discovered the formidable beauty of tusks and teeth, the emerging dexterity of opposing thumbs and the creative satisfaction of ivory carvings.

To the ancients, ivory became the poetic synonym of athletic strength and female beauty. For art and utility objects there was nothing quite like ivory. In its versatile history it has turned up in the form of holy crosses and deadly javelins; caskets for sacred relics and pillboxes for witch doctors; rosaries for prayer and dice for gambling; incense and cattle feed; altars and gaming boards; King Solomon's magnificent throne and George Washington's dental "bridge." Only recently has the tooth-borne object of this ancient art become subjected to scrutiny with the modern tools of dental science.

ORIGIN AND BASIC BUILDING BLOCKS

Not to be confused with what Pliny by way of contrast referred to derogatorily as "common bone," the ivory used most commonly in art and commerce[23] is the dentin of the elephant tusks (Fig. 1). These are the animals' middle incisor teeth, curved and pointed, and spread apart like "eye teeth" by the protruding proboscis, the "hand" of the beast.

In a broader sense, ivory can be dentin from other sources—from animals on land and sea, from little teeth to huge tusks: from the boar of the tropics to the bear of the Arctic; from the canines (eye teeth) of walrus and hippopotamus to the fabulous spiraling "unicorn" tooth of the narwhal; from the semicircular tusks of the mammoth, object of the caveman's art; and, last but not least, from the mandibular teeth of the sperm whale, object of the most recently introduced type of ivory carvings, the scrimshaw work that rescued the American whalers from boredom on their long voyages from the Pacific hunting grounds to their New England homes

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Observationes Additional Relative al Dimensiones del Crystallos de Osso

Summario in Interlingua

Studios electronomicroscopic del dimensiones de crystallos de osso indica que microcrystallos, in le forma de particulas de 30 per 50 A, es deponite latere a latere a transverso le sub-bandas intra le major periodo del fibrilla de collageno. In le curso del disveloppamento del crystallo, plus tal microcrystallos es deponite intra ille subbandas, con le resultante establimento de un relation de termino a termino con le preexistente microcrystallos intra le fibrilla. Quando iste disveloppamento es effectuate, le periodicitate del fibrilla de collageno es obscurate. Le direction del elongation de un crystallo particular es influentiate per le presentia de microcrystallos in le mesme fibrilla o in un fibrilla vicin. Isto significa que le crystallo es deflectite in le direction del plus proxime microcrystallo, establiente un relation de termino a termino in le nove plano e le nove direction. A causa del irregularitate de iste deflexiones, solmente un parte del crystallo pote esser observate, mesmo in sectiones que es orientate parallelmente al direction del fibrilla de collageno.

Il es probabile que le dimensiones del crystallos per se non differe multo in le apparentia de lor ultrastructura ab un specie al altere. Le autoritates es satis de accordo que le crystallos ha un ben-definite largor de inter 25 e 75 A, con un valor medie de 50 A. Super le base del hic-reportate constatationes il es possibile postular que le crystallos es componite de catenas de microcrystallos in disposition de termino a termino, comenciante con un longor original de circa 50 A e continuante sin limite apparente. Certe tal catenas esseva traciate usque a longores de 3,000 o 4,000 A. Il es possibile que le crescentia de crystallos es influentiate per le processo de refactionamento. Le resorption de osso resulta in le creation de spatio additional pro le nove deposition de crystallos.

("nerve") project their vitalizing protoplasmatic prolongations throughout the dentin matrix. The odontoblastic cell body is quite long, as cells go, cylindric in shape, several times longer than a red blood cell. But the tails of these cells, the so-called Tomes "fibers," are as long as the dentin is wide. This means, in the case of the elephant dentin—ivory, in other words—that these processes of cytoplasm are stretching out several inches away from the pulpal home base of the odontoblasts; equivalent, that is, to a dachshund with a tail longer than a baseball field. In contrast with other tail-wagging cells, such as the neurons of the brain, the odontoblastic tails have no room for play, confined as they are within the solid calcified wall of the dentinal tubules.

In elephant ivory the dentinal tubules turn in corkscrew fashion (Fig. 2) and make a number of almost right-angle, parallel dents in two planes. In reflected light, at low magnification, ivory shows broader bow-shaped stripes that cross in the arc of a circle and form curvilinear lozenge-shaped spaces by their decussations (see Fig. 1), giving to cut ivory an "engine-turned appearance, like the back of a watch." This is one of the best gross fingerprints of genuine elephant ivory, as pointed out by the British dental anatomist Sir Richard Owen,[23] when 100 years ago he spoke on "The Ivory and Teeth of Commerce" before the Royal Society of Arts.

In human dentin the dentinal tubules are very narrow (a red blood cell is three times as wide). But the tubules are so numerous that a microscopic cross section of dentin, when highly magnified, looks like a sieve with some 30,000 holes (cross-cut tubules) per square millimeter (Fig. 3). What makes elephant ivory take such a beautifully silky, glossy polish is the fact that the ivory tubules, while longer and even more numerous

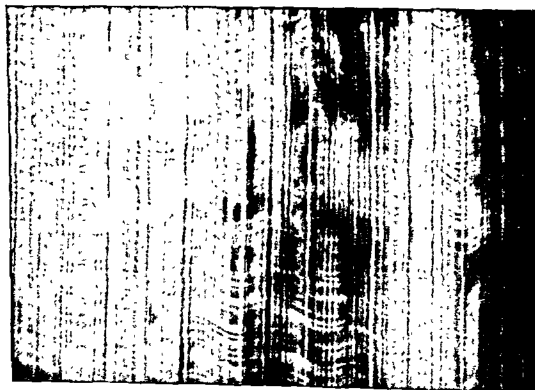


FIG. 2. Fluorescence of ivory shows organic matter concentrated along alternating growth rings (vertical bands), and spiraling dentinal tubules (wavy horizontal lines), which contain protoplasmatic extensions from the odontoblasts of the pulp. Using a ground section from the same tusk as Figure 1, this photograph was taken by Dr. A. F. Forziati, National Bureau of Standards, Washington, D.C.

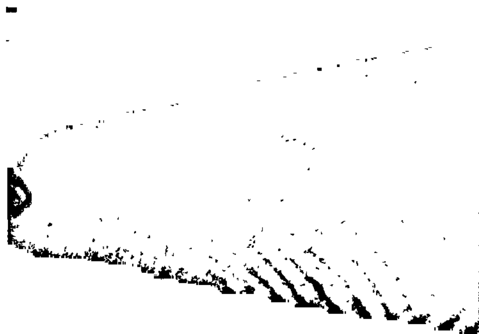


FIG. 1. Ivory "pie," cross-cut segment of elephant tusk, shows from left to right: the "nerve" center (obliterated pulp cavity) in core of tusk, fine circular (vertical) growth rings, like cross-cut wood, and wide decussating semicircular bands, the engine-turned reflection of cut ivory—"like the back of a watch"—fingerprint of genuine elephant ivory.

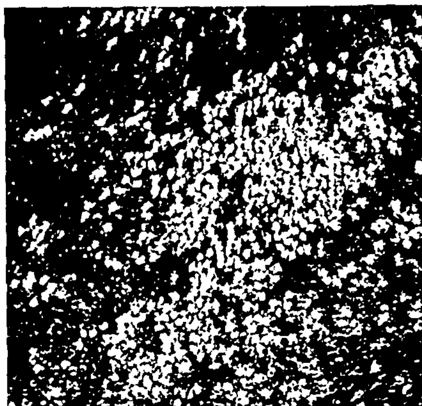
Pliny notwithstanding, ivory is the first cousin of ordinary bone,[12-14] in addition to being the big brother of the dentins.[23,26] With bone, the dentin substance shares a tendon-like organic framework, the collagen fibers, which, when broken and boiled, become jellied as gelatin glue. Interspersed between these reinforcing fibers is a more amorphous organic substance, yet to be labeled reliably. It has properties of a protein-carbohydrate complex and has been looked upon as a cementing substance (which it may not necessarily be), the mortar of the structure. The bricks, if one may pursue this unbiologic analogy, are made of inorganic lime salt. This building stone has certain physical properties similar to what in inanimate nature long ago was labeled as a *polyhedron apatite crystal,* literally "many-faced deceitful clear ice," prophetic to a point. The facts are far from clear when it comes to the apatite's counterpart in teeth and bones.[22] But we know that calcium phosphate is the principal inorganic salt of dentin, with smaller amounts of sodium, magnesium and carbonate, and a great variety of trace elements, ranging from the better-known fluorine to the man-made bone and tooth seeker of atomic fallout, radioactive strontium, from which caveman and mammoth, in blessed ignorance, were shielded so mercifully.

What makes dentin a super bone, despite several basic bony traits, is the fact that dentin is not pierced by the large channels and holes that house the osseous blood vessels and cells, and thus renders to bone its Swiss cheese microscopic morphology. By comparison, dentin is more like American cheese, loaded compactly with calcium and protein. This may have been appreciated intuitively by some of the Oriental tusk cutters, who are said to have been satisfied to keep the ivory sawdust in lieu of a salary as a tonic medicine, only to reinvest the fragments of their labor in the cow for the manufacture of more milk, better cheese and —for calves and children alike—stronger teeth.

A LIVING TISSUE

Though ivory contains no blood vessels or cells, nevertheless, its dense substance is pierced by minute dentinal tubules, through which the odontoblasts of the pulp

Fig. 5. "Milky-way" appearance of microradiograph of a ground section from aging human dentin. Dark holes are patent dentinal tubules; white zones are constellations of secondary mineralization, where the dentinal tubules are all but filled with mineral deposits (sclerosis), denser than the densest matrix of normal primary dentin. This preparation was made by John Nalbandian, D.M.D., Research Fellow at Harvard School of Dental Medicine. (× 1,000)



the tooth and the delicate nerves and blood vessels of the centrally located pulp. In hard-chewing Eskimos, this new dentin, formed in synchrony with the wear on the surface, crosses the gap below to form a protective ivory bridge, without which Eskimos ultimately would be chewing on a naked, painful nerve.

It is not surprising that we have thought of this protective secondary dentin forma-

Fig. 6. Single sclerotic dentinal tubule sectioned with a diamond knife and examined in electron microscope. The hole in center (cross-cut channel for the odontoblastic cell process from pulp) has been narrowed by deposition of extremely dense mineral salts (white doughnut). The adjacent dentin matrix shows bundles of collagen fibers, whose typical ultrastructural periodicity (640 A striation) is partly obscured by amorphous organic and crystalline inorganic matter. This specimen was prepared by Robert Frank, M.D., Chief of Research, Institut Dentaire, Faculté de Médicine, Strasbourg, France; *pro tempore* Research Associate in Oral Pathology, Harvard School of Dental Medicine. (× 20,000)





FIG. 3. Sievelike appearance is characteristic of seemingly solid normal dentin when a thin cross section is examined at very high magnification. The "holes" are cross-cut dentinal tubules, which contain the protoplasmatic cell processes extending from pulp. Decalcified section of dentin from rhesus monkey from India. (× 1,000).

than in man, are actually still narrower in diameter! In fact, the holes in the ivory "sieve" are barely within the resolving power of the standard optical microscope, about 1 micron in diameter. Moreover, the tubules are so close together that there is hardly more than a 1-micron sliver of solid ivory between each. In other words, ivory, like its dentin siblings in other animals, from rodents to man, is actually almost half holes, too small for man's eye to see; but unhappily, large enough—in man, at least—for the tiny, indigenous oral micro-organisms to detect and invade (Fig. 4), so as to produce tooth decay.[39,40,42]

A BIOLOGIC CHANGE WITH AGE

The dentin-forming cells, the odontoblasts, which are located along the internal wall of the dentin (in that hollow part of the tooth that contains the "nerve" or pulp), continue to function throughout life. Thus, within the "marrow" cavity of the teeth, if all goes well, there is elaborated what is called secondary dentin as the teeth are worn down. In this way, a safe distance is maintained between the chewing surface of



FIG. 4. Tooth decay, produced experimentally in the Norway rat, shows invading bacteria (darkly stained) penetrating the dentin from cavity on left, through the dentinal tubules (horizontal lines) and spreading sideways along the zone of appositional growth rings (vertical), the lines of least resistance. (× 500)



FIG. 8. Abnormal dentin formation is indicated by irregular odontoblastic layer (center) uneven border between pale dentin, pale uncalcified interglobular dentin zones and, running vertically, irregular incremental growth lines. (× 90)

Dr. John Nalbandian, Research Fellow in Dental Medicine, has repeated Gustafson's study on dental aging.[18,20] However, in his hands there was a greater spread in the biologic aging index of the teeth, so that the dental changes and the chronologic age did not coincide quite as well as in the Swedish study. This could mean either that the Boston teeth were more prone to pathologic states (which would tend to confuse the situation when superimposed on some of the genuine physiologic age changes) or that Bostonians were biologically more variable than Swedes—a concept one would not wish to defend on the basis of this limited evidence.

THE DRUM OF A METABOLIC KYMOGRAPH

The elephant's use of his tusks (for what man would need crowbars) takes its toll in ivory wear and tear. However, this loss is made up for by a complete rebuilding of new tooth substance, a biologic blessing not shared by man but characteristic of both elephant tusks and the incisors of our familiar rodents, large and small. In the common laboratory rat, for example, the incisor teeth are worn so rapidly on the chewing edge that the teeth would be worn down to the gum line in short order were it not for the fact that an equal amount of new tooth substance is elaborated on the root end of the same teeth within the jaw. Thus, every 6 weeks or so the whole semicircular incisor tooth is replaced. In other words, if one gets bitten by a 12-week old rat, the flesh is pierced by an incisal edge that was hidden in the jaw as the rear end of the same incisor 6 weeks before. As a result of this cycle of formation, calcification, attrition and re-formation, the rat incisor has become an important tool for research in various factors that influence growth and development (Figs. 7 & 8).

In 1911, the Austrian investigator Erdheim was the first to open wide this fruitful

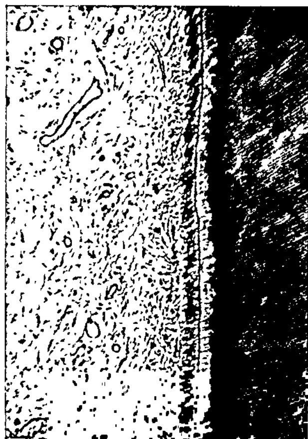


FIG. 7 Normal dentin formation as seen in a decalcified section shows, from left to right, loose connective tissue of the pulp (with nerves and thin-walled blood vessels), a vertical row of the elongated odontoblastic cells (center), a narrow zone of pale (uncalcifiable) predentin and then the dark-staining dentin, capable of attracting calcium salts. The fine, nearly horizontal dentin striations are caused by the dentinal tubules, which contain cellular prolongations from the pulpal odontoblasts. (× 90)

tion as a direct biologic response that would be produced only in the wake of wear and tear. According to Dr. Martin Rushton, of Guy's Hospital in London, it now seems that we may not have been entirely correct in this assumption [31] He was able to examine several adult teeth that, nonetheless, were free of the usual wear, because they were the protruding front teeth from persons with a so-called open bite, popularly associated with childhood thumb-sucking. The microscopic sections of these teeth showed that the dentin had undergone secondary changes similar to those that in the past had been attributed to the attrition of chewing. Now it seems instead that some of these secondary dentin changes are true biologic age changes, preparing the teeth, as it were, for a rainy day—whether that day comes or not. Perhaps this is as good an example as any of the cause-and-effect trap of biology and health research to which Dr. Alan Gregg, the late vice-president of the Rockefeller Foundation, referred in his story about the little girl who asked her mother why it was that the men selling pencils on the sidewalk were so prone to lose their legs.

Another even more important biologic dental age change can be seen in the root portion of teeth.[18-20] This change is more meaningful since we can rule out a number of variables that come from attrition and injury to the orally exposed portion of the teeth. This age change in the root dentin can be seen even with the naked eye if a tooth is slit down the middle along its long axis. Beginning at the root tips, the aging dentin assumes an increasingly glasslike transparency known as dentinal sclerosis (Figs. 5 & 6). Ultimately, in very old people, this secondary mineralization may extend throughout the better part of the root. This, I believe, is a more reliable physiologic aging index than the occlusal wear so long used in anthropology and forensic medicine for determination of the chronologic age at death.

Dr. Gösta Gustafson,[9] researcher at the Dental School in Malmö, Sweden, has worked out a system for grading the above and several other tooth changes, so as to provide a multifactorial point index corresponding to various chronologic ages. Using this grading system on a recent occasion, we were able to contribute one vital clue, the age of the victim, in a case of suspected murder. In my own laboratory, we have also used this "Sherlock Holmes" exercise in the histology course of students preparing for the Doctor of Dental Medicine degree. One of our recent graduates,



FIG. 10. Earthly saprophytes invading the dentin to cause postmortem canals in teeth. From prehistoric Greece: exhumed at Asine, Peloponnesus, Mycenae. (× 90)
(Sognnaes, R. F.: A.M.A. Arch. Path. 59: 563)

found, served to maintain normal differentiation of epithelial cells. In the absence of vitamin-A-containing food substance, it was demonstrated that there was a lack of normal differentiation (metaplasia) of the highly specialized enamel-forming cells. The cells reverted to squamous epithelium.[49] This metaplasia caused malformation of the whole tooth organ, especially the enamel, the "skin" of the teeth,[35] to which man owes his white smile or his cavities,[39] whichever the case may be.

A CLUE TO THE VOIDS IN IVORY ART

Though the teeth are so extremely prone to decay during life, generally they are the best-preserved parts of the body after death, as has been known to the scientific advantage of anthropology for a long time. Why, then, as the Bible predicted (Amos 3:15), should the ivory houses perish? While, presumably, some of the ivory building blocks were carried away by the human hands of barbarian plunderers, we now know that other culprits, waiting in the ground, were fully capable of consuming the remaining fragments. What is attractive to dogs and good for cattle feed on top of the ground is equally appetizing to subterranean saprophytes.

In a recent study[37] we found that these earthly *postmortem invaders, probably fungoid* in nature, had made a microscopic spongelike shambles of several otherwise good anthropologic tooth collections from various parts of the Old and the New Worlds (Figs. 9 & 10). In some burial sites, rootless shells of dental enamel were the best human remains—and sometimes the *only ones—scattered,* like so many Hollywood porcelain crowns, among the tools of early man (Fig. 11). How can the saprophytes tell enamel from dentin, ivory and bone, all of which contain the same mineral, the apatite crystals? *Evidently,* these deceitful inorganic bricks are only the appetizers as it were. What the saprophytes really are *after* is obviously the protein-loaded connective tissue collagen. The seemingly unattractive enamel has an organic framework of epithelial origin.

Unhappily, the tusks of adult elephants—and the objects of art *and utility* fash-



Fig. 9. Prehistoric human molar tooth, exhumed from Greek soil, appears to be intact on the surface (*left*), but a roentgenogram (*right*) indicates that the internal root dentin is no longer solid. (× 4) (Sognnaes, R. F.: A.M.A. Arch. Path. 59:562)

field of rodent incisor research. His "discovery" of the incisor was incidental to his pioneering studies on experimental endocrinology. By surgically removing the parathyroid gland, which was to exhibit such an important role in calcium metabolism, he found that the rat incisors responded promptly by the formation of a poorly calcified layer of dentin immediately after hormonal depletion and that, when the transplanted gland had taken hold, new dentin of normal quality was again laid down. On the basis of this layer-by-layer response of the tooth dentin—resembling rings in a tree after good and bad seasons—he concluded that "the dentin, as it is being apposed, rolls on slowly and uniformly like the drum of a kymograph, on which are recorded the vacillations of calcium metabolism in a most accurate and readable manner."[12]

For that same reason, the dental organ has become an equally profitable research tool in studies of the role of vitamins and trace elements in growth and calcification.[10,34,41] Before the dawn of nutritional science, it was known that teeth became loose in scurvy-ridden sailors. Teeth are anchored to the jaw bone by the tendonlike periodontal fibers of collagen similar to those of ivory.

Using the incisors of guinea pigs as target organs, studies of the effect of scorbutic diets were initiated in the early twenties by Dr. Percy Howe, then director of The Forsyth Dental Infirmary, Boston, and followed by life-long collaboration with Dr. Bert Wolbach, former professor of pathology at Harvard Medical School. They instituted deficiencies in what was at first called an antiscorbutic accessory food factor (now vitamin C) for various periods of time, after which the missing food substances were replaced. It turned out that the incisors behaved as sensitively to these nutritional factors as they had to hormonal influences in Erdheim's work. In the course of a few weeks, this growing tooth tissue could be studied through all the intricate processes of cellular differentiation and fiber formation.[48] Furthermore, because the teeth originate from two germ layers, the dentin coming from connective tissue and the enamel from epithelial tissue, observations could be made not only of the effect of vitamin C, which, it turned out, served primarily to maintain normal connective tissue (collagen) formation, but also of vitamin A, which, they



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Unhappily, the tusks of adult elephants—and the objects of art and utility fash-

ioned from ivory—have no telltale enamel to leave behind. Thus, depending on the type of soil in which the ivories happen to become buried, they may become virtually destroyed, as in certain ancient graves from Guatemala to Finland; or the ivory may be well preserved, as in the dry, sandy soil of certain parts of Egypt, and in the chilly ground of Siberia, where one has found intact mammoth, ivory tusks and all, the last meal undigested in the stomach, as though the animal had fallen through thin ice into a deep freeze.

Intermediary stages of ivory destruction have been found. One of the earliest examples is the so-called Venus of Brassempouy, a carving by cave man of cave woman, who appears moth eaten and has almost completely lost her head. More recent fragments, such as the crumbling early ivories found in Samaria in the 1930's, may be, in fact, remnants of the ivory house thought to have belonged to Sargon II, King of Assyria, from 722 to 705 B.C. There are probably many other gaps in the ivory art history that we never shall be able to fill. Yet it is remarkable that so many ivory objects of art and utility have "survived" this saprophytic consumption, so as to form an almost uninterrupted chain through man's cultural evolution.

VARIATION IN QUALITY

Long ago men of philosophy, art and commerce were aware of the fact that ivory was not always what it ought to be; that its quality of structure sometimes could be more Swiss than American, so to speak. According to Pliny and Philostratus, the tusks of the "stupid and idle" elephants inhabiting the marsh were porous and hollowed out into many "cavities" compared with the homogeneous ivory structure of the elephants living in the mountains and on the plains. The latter, it was claimed, had the largest, whitest and best ivory tusks of all. More than that, in these favored beasts there was detected a coincidence between dental and mental superiority: for these animals, the ancients maintained, were fond of learning tricks; they could write, dance, and swing and "sway to the sound of the flute"—an ivory flute, I presume.

If we think the supply of tooth-building

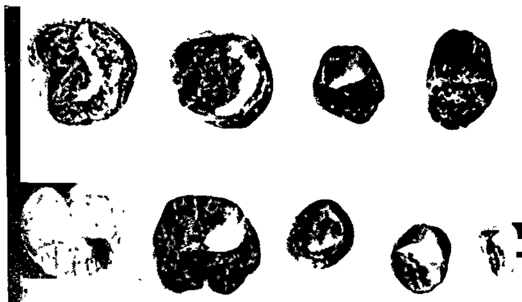
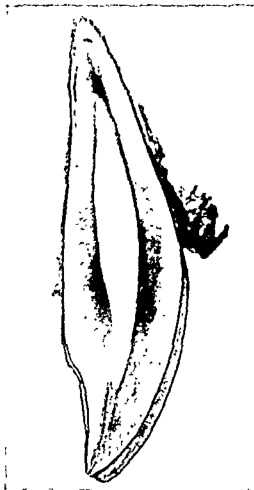


FIG 11 Enamel shells from exhumed American Indian teeth look like porcelain Hollywood crowns because of postmortem destruction of the ivory core of the teeth.

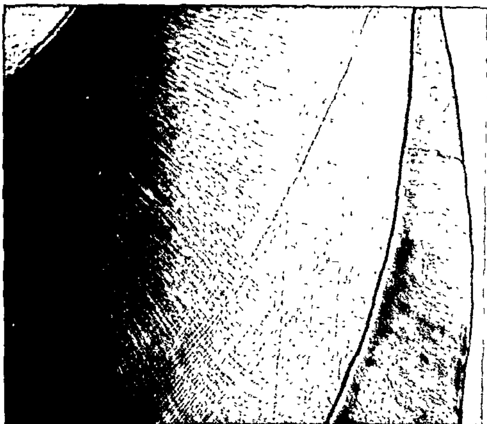
FIG. 12. Incisor tooth of rhesus monkey from India shows, around hollow pulp cavity (*center*), the dentin core, which forms the bulk of tooth, and is covered (*below*) with a layer of enamel over the crown and (*above*) a coating of bony cementum round the root. (× 6)



elements is critical in the case of our milk-fed, sunbathed, cod-liver-oiled children, consider the problem of the thick-hided pachyderms, who have to rely on calcium-poor grass, leaves, fruits and shady trees to yield, after enormous digestive losses, enough calcium, phosphorus and protein to build a couple of hundred pounds of teeth, not only once like man, but again and again. For, as noted above, while the ivory tusk is worn down on one end, it is being rebuilt anew from the other throughout life.

In general, elephants prefer to roam in the shade of the forests. This is thought to be particularly true of the Indian species, whereas the African elephant has been known to wander into the stronger sun of

FIG. 13. Structural perfection of monkey tooth is indicated by beautifully calcified dentin with delicate growth rings (*center*) and homogenously formed enamel (*right*), the "skin" of the tooth. A small pie-shaped portion of the "nerve" or pulp tissue, lined by a row of dentin-forming cells (odontoblasts), is seen in upper left-hand corner. (× 55) (Sognnaes, R. F.: Science, Dec. 18, 1959)



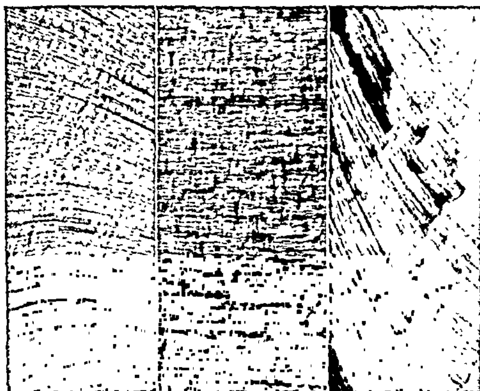


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extremely high altitudes. There are many exceptions to the rule of thumb of the ivory trader, according to which ivory from equatorial West Africa is better than that from the low-leveled east and north; but it is interesting that ivory carvers, even in far away China, have considered African ivory to be superior to that from the Indian elephant.

Today, we know a good deal more about what makes for bigger teeth and better dentin. Size and shape of teeth may not be predetermined by genetics alone, as is generally believed. Dental research workers at the *University of Toronto Dental College* recently were able to show that the size and the shape of rat teeth could be altered by the nutritional background of the animals, the dietary levels of vitamin A and fluorine being two of the factors demonstrably involved.[6] Differences in the dimension and the configuration of the grooves in the chewing surfaces of the teeth, whether of genetic origin or nutritional, may also explain, in part, why some are resistant and others more susceptible to tooth decay.[8,11]

In England, Lady Mellanby has demonstrated that the microscopic qualit tin can be altered drastically by th ing property of the diet.[15] In puppi on diets with unfavorable propo mineral salts and lack of vitamin cheese dentin was made. In child the most homogeneous mineralizati teeth, fewer cavities were found.[1] United States, the quality of hum has been correlated with latitude exposure. Going north from the Gulf to the Canadian border it h shown that for each latitude ther increase of 15 decay-ridden teeth *children.*[17] *In localities in the Unite* with an average of as much as 3,0 of annual sunshine, 40 per cent le decay has been found than in pla only 2,000 hours of sun exposure p

While this is guilt by association is a fact that human ivory is exc prone to poor calcification. Man's dentin stands in sharp contrast w homogeneously calcified teeth of the monkey (*Figs. 12 & 13*) We have man's trouble all the way back to t man.[18] In between man and monke

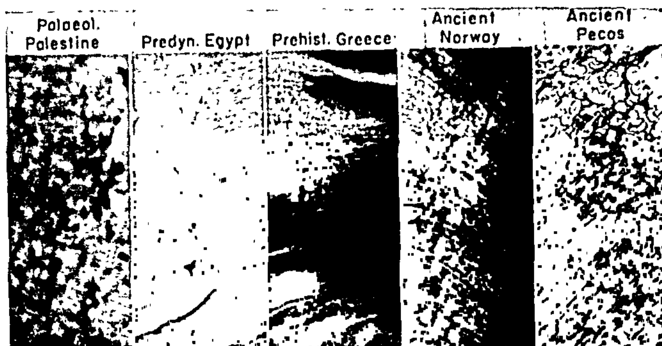


FIG. 15. Homo sapiens dentin, from prehistoric and ancient man, not only has the faulty granular structure of the lower anthropoids but often shows dark microscopic slits of completely uncalcified interglobular dentin spaces. (× 60).

the terrestrial apes[13]—gibbon, gorilla and chimpanzee (Fig. 14)—whose dentin, while inferior to that of the rhesus monkey basking in India's treetops, is superior to the dentin of homo sapiens (Figs. 15 & 16).

A PRACTICAL PROBLEM OF ART

Unlike many other raw materials for art and utility objects—ranging from pottery to plastic—ivory cannot be hammered, melted, cast or baked into new form by any tools

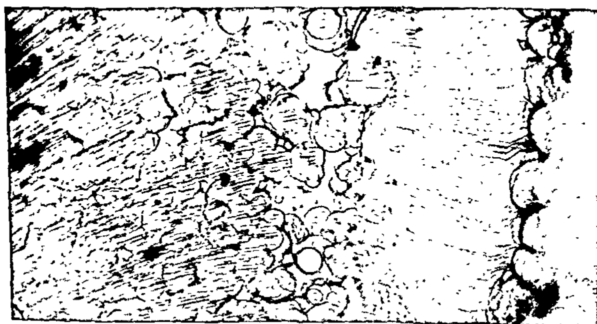


FIG. 16. Pecos Indian incisor with extremely poor structural quality at the opposite extreme of the monkey incisors (see Figure 13). Under the abnormally folded junction between enamel (*right*) and dentin, there is only a narrow zone of solid ivory tooth substance, followed by numerous soft spaces where the calcifying dentin globules have failed to fuse. (× 150)

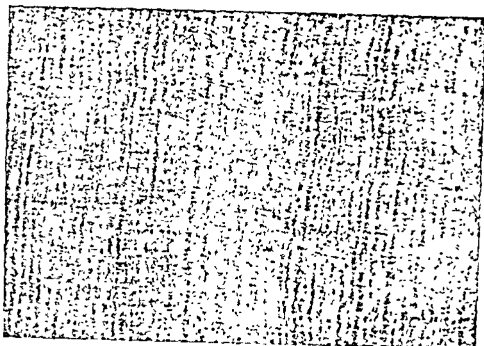


Fig. 17. rhythm of ivory is see lines) when a of ivory is in the optic scope by light. Minut tubules run h through ivo (× 100)

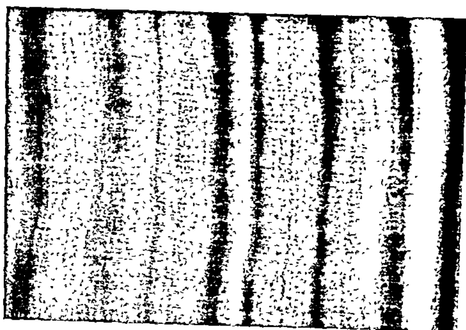


Fig. 18 Mi graph of ivory by shooting so (Grenz rays) thin ground placed on a fine photographic fi vertical band alternating la high (white) (dark) density cification. The horizontal lines dentinal tubule run from the the surface of t tin substance. (

known to modern man. Yet, the earliest literature on ivory art suggests the possibility that the ancient artists may have had some tricks of the trade whereby they succeeded in softening, bending and rehardening ivory slabs into desired shape beyond that provided by nature. To this end, the enthusiastic artist monk, Theophilus,[46] in his treatise *Diversarum Artium Schedula,* presumed written some 900 years ago, described the use of a mixture containing sulfate of potash, fossil salt and vitriol, ground in a brass mortar and mixed with sharp vinegar a buffered solution of acetic acid, quicker for softening ivory than the urine used by Eskimos,[21] was obvious as drastic for softening ivory as the and phosphoric acid treatment r mended in 20th-century references t subject.[47]

But what about the rehardening of ened ivory? This is a question that stil fles science. If, through trial and error, ancient ivory artist really succeeded in

ing this problem, he certainly must have taken the deeper secrets to his grave. That efforts were made *in this direction* is *indicated* by a prescription in the 12th-century English manuscript *Mappae Clavicula*, by Phillips[26]:

Quicklime	2 parts
Pounded tile	1 part
Torn Tow	1 part
Oil	1 part

The above-mentioned ingredients were to be mixed with a lye made from elm bark before use; that is, for the soaking of softened ivory. Chemically, this alkaline solution of calcium oxide, silicate and polysaccharide did have ingredients that on the basis of later information could be expected to influence the mineralization of ivory; but, to my knowledge, no modern artist or scientist has duplicated the "experiments" of the ancients. Yet, the German ivory art historian Pelka[27] has maintained that (author's translation) "the definite manner in which these references have appeared makes it probable that the ancients, in fact, did possess such a technic, the knowledge of which has become lost."

On the basis of some current research in our own laboratory, we have come across one structural feature of ivory that in part could account for the artists' reshaping of ivory slabs. We have found that, when thin slabs of ivory are placed on a fine-grained photographic emulsion and shot through by soft x-rays (Grenz rays), even good healthy ivory (the "American cheese" variety) is not as homogeneously dense as we thought (compare Figs. 17 & 18). On the contrary, our microradiographs indicate for the first time that ivory is divided into nearly equal alternating layers of high and low microdensity (Fig. 18), somewhat like plywood. Thus it is possible that the ancient ivory artist may have achieved a partial demineralization of the already semisoft alternating zones, after which the structure could be more readily bent, squeezed together and dried in a new and different external shape. At that point the artist may have let well enough alone, with the hard layers about as hard as before and the semisoft, low microdensity layers (Fig. 18) a little softer, but compressed, dehydrated and condensed. And yet, there may be more to it than this, and better explanations may evolve as we find more basic answers to the remineralization problem.

A THEORETIC EXPLANATION OF SCIENCE

For many years it has been common practice for the dentist to line the acid-soaked softened dentin in the bottom of deep cavities with a dash of an alkaline mineral salt (calcium hydroxide), in order to induce the formation of a hardened dentin bridge across the ivory gap. More often than not it works. Nobody knows exactly why, any more than we know why other tissues calcify and harden, be it bones, arteries or kidneys. Nor, for that matter, do we know what prevents us from calcifying all over, turning into statues of salt.

Clearly we are dealing with interrelated problems requiring correlated approaches. No longer are teeth and bones the scientific concern of anthropologists, dentists and orthopaedic surgeons alone. It is noteworthy that virtually all fields of the healing arts and biologic sciences have been represented at the annual Gordon research conferences on chemistry, structure and physiology of bones and teeth, which, since their beginning in 1953, have attracted an overflow number of participants each year. Toward similar ends abroad, there recently was organized the Bone and Tooth Society of Great Britain. At the 125th annual meeting of the American Association for the Advancement of Science, held in Washington on December, 1958, the Section on Dentistry, together with the Sections on Medicine and on Zoology, sponsored a 3-session symposium on calcification in biologic systems, during which discussions ranged over the whole animal kingdom,



FIG. 19. Autoradiograph of a calcifying monkey molar tooth, prepared a few days after intravenous injection of radioactive phosphorus (P^{32}), shows how the young new zone of dentin along the wall of the pulp cavity (lower white "hot" line) has attracted a high concentration of phosphate salt. The central dark M-shaped zone is dentin largely calcified before P^{32} injection, hence not so "hot" The white hat on top is enamel, the skin of the tooth, where the phosphate crystals continue to grow to much larger dimensions than in dentin and bone. (× 7) (Sognnaes, R F., Ann New York Acad. Sc. 60:564)

from lobster claws to human teeth.[43] While there is as yet no unified concept to account for the mechanism of mineralization in living organisms, much will be gained by the widespread multidisciplinary interest that now is being focused on the problem here and abroad.

Already, as a result of this interest in calcification, science is getting excitingly close to an explanation in theory of what to some ivory artist may or may not have been an accomplished fact Basically, one of the most stimulating conceptual schemes grew out of the pioneering observations of an orthopaedic surgeon, Dr. Robert Robinson In 1952, working at the University of Rochester, he reduced bone and dentin to minute fragments and noted, by means of electron microscopy, that the submicroscopic inorganic apatite crystals appeared to line up in *a precise periodic manner along the reinforcing* organic fibers [29]

Subsequently confirmed by more refined technics,[30] this finding may be considered a scientific breakthrough that helped to shape a key question: What is the chemical attraction of this organic matrix of ivory, teeth and bones that makes calcium salt normally settle down just there and not all over the body? This problem has been subject to numerous studies, reviewed in detail elsewhere.[1,4,14,41,43,50] We have mentioned already that the organic framework of ivory shares with other dentin and with bone a reinforcement of collagenous fibers. These fibers can be split up into their macromolecular units by means of acetic acid. Upon subsequent exposure to certain salts and polysaccharides, these units can be made to reunite. Finally, the reconstituted collagen can be made to serve as nucleation center for mineral salts.

Recently, such nucleation of calcium phosphate on reconstituted collagen fibers has been achieved in the test tube by researchers at Massachusetts Institute of Technology.[6] Examined in the electron microscope, the process appears to be similar to that which occurs in living bone and dentin. The apatite crystals *line up like* beads on a string in a precise periodic way along the collagen subunits. If that is so, one may wonder why calcium does not settle down on the collagen fibers everywhere, in tendons, skin, cartilage and other sites. One reason may be that these and other normally uncalcified tissues tend to contain higher quantities of an acidic carbohydrate-protein complex (mucopolysaccharide) that may help to maintain certain soft tissues, and even parts of the skeletal and the dental tissues, permanently or transitionally in an uncalcifiable state.[36] Cartilage has a collagenous fibrous framework; but it is extraordinarily rich in acid mucopolysaccharide and may remain uncalcified throughout life. The same situation can obtain in very limited microscopic areas of adult dentin and bone, *whereas, in young dental enamel, such* a situation appears to be transitional. For, in enamel, one may detect a cartilaginouslike



FIG. 20. Electronmicrograph of ivory from Indian elephant tusk shows the minute pencil-shaped ultrastructure of the "deceitful" apatite building blocks common to dentin and bone. These dense inorganic crystals of calcium phosphate, here the size of exclamation points, can barely be made out within the thinnest transparent slivers (center and lower corners) of the section. (× 340,000)

matrix reaction[36] only during a short period of development; namely, when uncalcified pathways still remain open. The subsequent influx of phosphates[44] into the depth of enamel ultimately makes it the densest and the hardest of all biologic structures (Fig. 19), being then almost devoid of polysaccharide and H_2O.

In addition to variations in polysaccharide content, there now appear to be potential differences in the chemical reactivity of the types of fibrous protein found in soft tissues and hard tissues. Last year it was reported by Solomons and Irving,[45] working at the Joint Dental Research Unit at the Witwatersrand University in South Africa, that dentin collagen, unlike its soft-tissue counterpart, contained more freely reactive chemical radicals (e-lysyl and hydroxy-lysyl amino groups). These free radicals may serve as a suitable seeding template for the growth of the inorganic crystals (hydroxyapatite). In ivory (as in bone) these pencil-shaped structures require the new vistas of the electronmicroscope — and maximum magnification at that—to be visualized (Fig. 20).

Notwithstanding this progress, it is quite possible that these new concepts of mineralization are still too connective-tissue (collagen) centered. For just as these seemingly satisfying schemes have come to light, there has appeared a report on the demonstration of apatite "bone" salts in a system of unicellular organisms.[24,25] We have observed similar "bone" crystals in dental calculus (tartar).[7] Here neither collagen nor other *extra*cellular fibrils, but probably some more fundamental chemical radicals, must have served as the nucleation center for mineralization. Recently we have found that the epithelial cells that give rise to the dental enamel (ameloblasts) elaborate nonstriated organic fibrils capable of nucleating hydroxyapatite crystals.[5]

Whatever, then, is the biologic mechanism or chemical, molecular specificity that gives ivory and other dentin, bone and den-

tal enamel such great affinity for minerals, we have not ruled out the possibility that the ivory artist, through trial and error, may have stumbled on the secret of calcification, though as yet we have not fully succeeded, through modern science, to apply our theories to practice, either in the promotion or the prevention of calcification.[43] When, through further research, we do succeed, it will not be for the artistic molding of elephant ivory, but for more scientific control of calcification in biologic systems.

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Le Substantia Eboree e Altere Typos de Dentina

Summario in Interlingua

Ebore es un specie de "fratre major" de dentina, le "prime cosino" de osso. Illo possede in commun con altere tissus dur mesodermal un orientatissime struction de fibras de collageno, un diffusemente dispergite substantia fundamental de mucopolysaccharido, e densemente paccate crystallos inorganic de hydroxyapatite que exhibi le mesme characteristicas electronomicroscopic como le correspondente crystallos de osso. Ben que disproviste del canales e lacunas que alberga le vasos sanguinee e le cellulas de osso, ebore—nonobstante—es alimentate via un multitude de micrissime tubulos de

circa 1 micron de largor que contine le prolongationes protoplasmatic del odontoblastos le quales revesti le pariete del centralmente locate pulpa o "nervo". *Durante su disveloppamento le matrice dentinal exhibi* un sensibilissime responsivitate al influentias que affice le mechanismo de *calcification* (un facto initialmente *evidentiate per le* experimentos de transplantationes parathyroide de Erdheim in 1911). In responsa al *processo de invetulation* physiologic (e accentuate per excessos de *uso e abuso*), le tubulos dentinal es *oblitterate per mineralisation* secundari. Iste processo *de sclerosis* ha essite demonstrate per *roentgenomicroscopia* e electronomicroscopia e *representa* probabilemente un del plus fidel *indices del* ver invetulation biologic del dentes. Le grande variabilitate *del qualitate* de ebore (ben cognoscite al eboreros *e eboristas del* antiquitate) es melio illustrabile *in le specie* del primates. In dentes human, depost tempores prehistoric e usque al presente, le dentina se monstra inclinatissime a *remaner* imperfecte in su calcification. Isto contrasta marcatemente con le dentes del moderne simias del generes vegetante in le coronas del arbores de India, durante que le *qualitate* del dentina de simias terrestre occupa un position intermediari. Ab iste puncto de vista le simias terrestre es inferior a lor cosinos arboricole sed superior a homo sapiens.

Le moderne scientia ha succedite a clarificar varie aspectos problematic del arte de ebore del antiquitate. Objectos de arte e utensiles ex ebore forma un quasi ininterrumpite catena a transverso le historia evolutive del cultura human. Tamen, il ha certe lacunas in le historia del arte de ebore, e istos—*si ben* como le prediction biblic que le domos de ebore va perir—es explicabile per le demonstrate appetito de saprophytos subterranee (probabilemente fungos) *pro le matrice* de collageno ca ebore e *altere typos de dentina.* iste destruction biologic es simil occurre in le resorption de osso i tia de osteoclastos, in tanto qu *processos le ingredientes inorgani* del substantia dentinal es destr taneemente sin le occurrentia de *cative* gradiente de dismineralisati

Studios per medio de microra a alte resolution ha resultate in le tion que le apparentemente solid tia eboree es dividite in quasi equa bassissime e de altissime microden nove constatation explica possibil apparente capacitate del sculptor de re-conformar ebore per flecte mente dismineralisate placas de ist a in le forma desirate, multo in limites providite per le natura. U plus interessante ab nostre current de vista es le apparente relation effortios del ancian artistas de remi specimens de ebore mollificate e e nostre tempore de initiar calcific vitro. Es presentate exemplos d concoctiones de ancian artistas—d in documentos de novem seculos ret pare haber continite ingredientes (emplo acido acetic, polysaccharidos sales de calcium, etc.) de affinitate altemente purificate substantias chi es usate per moderne investigatore dissolution de tissus conjunctive e le tion de sales mineral in reconstituit geno.

In le fin final, le comprension mechanismos va illuminar non solm natura del processo calcificatori norn etiam le factores que servi a prev deposition anormal de concretiones lose in e circum le vasos de sangu renes, e le dentes.

7

Microchemical and Biophysical Studies of Normal Human Compact Bone Tissue

With Special Reference to the Organic Component

B. ENGFELDT AND J. STRANDH*

INTRODUCTION

The mineralization of microscopic structures in bone tissue has been studied thoroughly during recent years. In these investigations, biophysical technics — above all x-ray microscopy — have been used in order to get quantitative information on the distribution of bone salts.[1] Recently microchemical methods also have been adopted for the study of microscopic structures.[12] From the results obtained by these various technics we now have a fairly good knowledge of the distribution of mineral salts in human bone tissue. However, similar information concerning the organic component of bone tissue is scarce.

The histology of the organic component of bone tissue is well known.[11] It is composed of a fibrillar protein — collagen — which comprises more than 90 per cent of the dry weight of the organic part of the tissue. The collagen fibers are organized in bundles of varying size. In adult human bone tissue, these bundles constitute lamellae in which the collagen fibers have a high degree of orientation It is thought generally that such lamellae are differentiated from the surrounding lamellae through the orientation of their fibers.

Apart from the collagen fibers, the organic part of the bone tissue also contains so-called cementing substance. The exact composition of this material is not known, but it contains an acid mucopolysaccharide. Chondroitin sulfate has been demonstrated in small amounts in bone tissue,[9] and recently the presence of keratosulfate has been described.

We report here some results obtained by microchemistry, x-ray microscopy and microinterferometry on the distribution and the organization of the organic component of microscopic structures in normal human compact bone tissue.

MATERIAL AND METHODS

A study was made of bone tissue from children and adults without bone disease. Compact bone from the diaphyses of the femur was cut into thin transverse slices, and these slices were ground on glass plates to a thickness of 100 to 300 μ. The sections were subjected to x-ray microscopy, using a Philips diffraction unit equipped with an x-ray tube utilizing a Cu anode as radiation source. The sections were placed in close contact with a fine-grained photographic emulsion (Kodak spectroscopic plate No. 649) and exposed to x-rays generated at 24 kw. After processing, the pictures thus obtained were enlarged by photomicrography. The x-ray absorption caused by the specimen in this procedure reflects the dis-

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ition of mineral salts, the contribution of organic material to the absorption being igible.[4,5] Then the specimens were decal-d in Versene at pH 7, taken through the hols to cederoil and then embedded in wax. This material was cut on a slid-microtome in 5-μ-thick sections, which floated on to a photographic plate of same type as mentioned above. Next, e sections were examined with ultrasoft ys in order to get information on the ibution of dry weight throughout the te.[7,8] Some of these sections were utilized rward for microinterferometry. The sec-s were studied in a Cook interference roscope. The principle of dry mass rmination by microinterferometry has 1 given by Davies and Wilkins.[3]

1 order to study extremely small details undecalcified bone, slices were ground to ickness of 10 μ or less and then were ed on a photographic emulsion and ex-d to x-rays generated at 6 kv. After-d these sections were decalcified in Ver-and again placed on a photographic ilsion and exposed to ultra-soft radiation the wavelength region 10 to 50 A in er to study the dry weight of certain ictures The technic of preparing sam-for this type of work is discussed in ail in another article.[2]

For the microchemical studies the follow-procedure was adopted. Guided by a roradiogram of a 300-μ-thick plano-allelly ground transverse section of femoral bone tissue, haversian systems of varying degrees of x-ray absorption and selected portions of lamellar bone were dissected out. The dissected haversian systems were separated according to their degree of x-ray absorption into 4 groups. Each group consisted of 12 to 18 portions of haversian systems. A 5th group was made up of a comparable amount of lamellar bone tissue.

Prior to the chemical analysis, the volume (approximately 0.2 μl.) of the bone tissue in each group was determined by planimetry of the microradiograms of the dissected microscopic structures and calculations based on the known thicknesses. The bone tissue then was hydrolyzed with hydrochloric acid, after which microchemical analysis was performed to determine the amino-nitrogen and the phosphorus content in the separate hydrolysates.

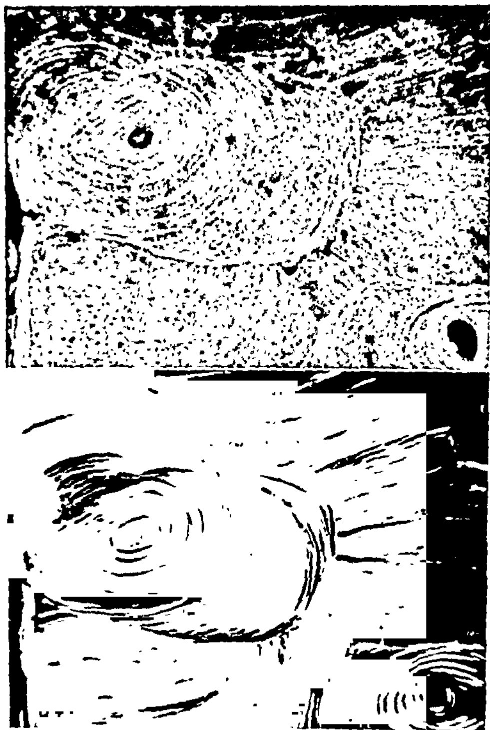
The amino-nitrogen that was taken to represent the organic components of bone tissue was determined according to a modification of the ninhydrin reaction described by Moore and Stein.[10] The phosphorus was estimated according to Youngburg and Youngburg[13] in 1930. For further details on the procedure of microchemical analysis of bone tissue the reader is referred to Strandh.[12]

RESULTS

Using microchemical methods, it was demonstrated that the amount of amino-nitrogen per unit volume was practically

MOUNT OF AMINO-NITROGEN (μG.) AND PHOSPHORUS (μG.) PER UNIT VOLUME (μL.) NE TISSUE IN MICROSCOPIC STRUCTURES OF VARYING DEGREES OF X-RAY ABSORPTION, DISSECTED FROM A TRANSVERSE SECTION OF NORMAL HUMAN COMPACT BONE

Fig. 1. (*Top*) Microradiogram of a 5-micron thick transverse section of decalcified compact normal bone tissue from femur of human adult. 10-50 A radiation. Variations in x-ray absorption indicate differences in dry mass per unit area. Light areas have a higher dry mass than dark. To the left is seen a haversian system with concentrically arranged areas of alternating dry mass. A similar pattern also appears in the surrounding interstitial and lamellar bone. (× 200) (*Bottom*) Photomicrograph, polarized light, of the same area as shown above, demonstrating the arrangement of the collagen fibers. (× 200)



constant throughout the transverse section of normal human compact bone tissue. As can be seen in the table on page 64, there are no significant variations in the distribution of the organic components, regardless of the degree of x-ray absorption of the undecalcified microscopic structures.

X-ray microscopic and microinterferometric studies of the distribution of dry mass in demineralized sections of adult human compact bone has given us the following information:

Comparing the dry mass per unit area within single haversian systems, it has been shown that the organic material is inhomogenously organized (Figs. 1, *top*, and 2, *top*). Thus, in the haversian systems, areas with alternating high and low dry mass are seen arranged concentrically round the haversian canal. A similar pattern is also demonstrated in interstitial and lamellar bone tissue (Figs. 1, *top* and *bottom*). When studying decalcified coarse fibrillar bone from newborns, small irregular ...

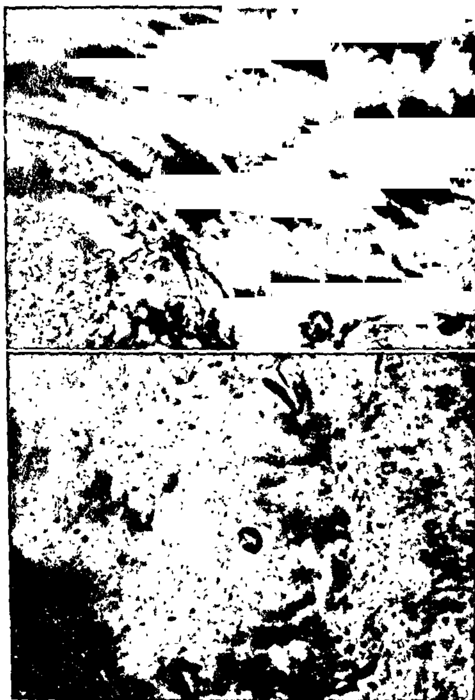


FIG. 2. (*Top*) Microinterferometric photograph of decalcified compact bone tissue showing part of a haversian system. The interference bands passing the bone tissue produce a zigzag pattern indicating variations in dry mass per unit area in different lamellae. To the lower left, part of the soft tissue of the haversian canal is seen (× 667) (*Bottom*) Microradiogram of a 5-micron thick transverse section of decalcified compact bone from a newborn baby. 10-50 A radiation. In the middle, a primitive haversian system is seen. Small variation in dry mass per unit area can be observed. The lamellar pattern found in adult bone tissue is lacking. (× 200)

tions in dry mass without the lamellar arrangement seen in adult bone could be observed (Fig. 2, *bottom*)

DISCUSSION

If macroscopic parts of bone tissue, such as transverse sections about 300 μ thick (weighing about 35 mg.) of normal human compact bone from the diaphyses of the femur, are analyzed for total amount of phosphorus and nitrogen, no significant variations can be observed in adjacent sections. However, the adoption of x-ray microscopic procedures for the study of the distribution of mineral salts throughout a transverse section of bone tissue has shown varying x-ray absorption, indicating appreciable variations in content of bone salts in different microscopic structures. The microradiograms of compact bone tissue show that most haversian systems have a high degree of x-ray absorption. However, single

systems with a very low absorption are observed, as well as some intermediate forms. The interstitial bone and the circumferential lamellae have a high x-ray absorption. Microchemical analysis of these structures showed that some of the haversian systems—i.e., those with the lowest x-ray absorption—had a degree of mineralization that was more than 25 per cent lower than that of interstitial bone (cf. Strandh[12]).

When the amino-nitrogen representing the organic component was estimated per unit volume in different microscopic structures, no significant variations could be demonstrated. Thus, although there are considerable variations in mineralization between microscopic structures on this level, the organic component has been shown to be evenly distributed.

When a single haversian system is studied in more detail, variations in mineralization, as well as in distribution of the organic material, can be demonstrated. As shown by Amprino and Engström,[1] an undecalcified haversian system with a low x-ray absorption has a decreasing degree of x-ray absorption from the haversian canal toward the periphery. Furthermore, in very thin transverse sections, concentrically arranged areas with alternating low and high x-ray absorption have been observed, indicating variations in mineralization.[6] In the present investigation, studies of decalcified sections of haversian systems have shown the organic component to be distributed in a lamellar way with alternating low and high dry weight. The arrangement of these lamellae are similar to that observed in undecalcified sections. This type of variation in mineralization and in distribution of organic material has also been demonstrated in the interstitial bone tissue.

As more than 90 per cent of the organic material is made up of collagen, the observed variation in dry mass probably represents variation in collagen content within these structures

From the observations mentioned above it may be concluded that there is a connection between the degree of mineralization and distribution of collagen within these microscopic structures.

SUMMARY

Microchemical, x-ray microscopic and microinterferometric studies of the distribution of the organic component of normal human compact bone tissue have been performed. It has been shown that when comparing different microscopic structures, such as haversian systems, no appreciable difference in content of organic material occurs. However, within a single haversian system, considerable variation in dry mass per unit area, organized concentrically, can be demonstrated. The same is true of the lamellar bone.

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Studios Microchimic e Biophysic de Normal Tissu Ossee Compacte ab Humanos, con Referentias Special a Su Componente Organic

Summario in Interlingua

Esseva effectuate studios microchimic, roentgenomicroscopic, e microinterferometric del distribution del componente organic in normal tissu ossee compacte ab humanos. Esseva trovate que in le comparation de differente structuras microscopic, per exemplo le systemas haversian, nulle appreciabile differentia occurre con respecto al contento de materiales organic. Tamen, intra un sol systema haversian, considerabile variationes, concentricamente organisate, pote esser demonstrate in le valores del massa sic pro unitate de area. Le mesme constatation es valide con respecto a osso lamellar.

8

Crystal-Collagen-Water Relationships in Bone Matrix*

ROBERT A. ROBINSON, M.D.†

A few points are quite clear concerning the development of bone tissue under normal conditions.

Extracellular bone matrix has to be present before mineralization occurs, since the mineral would have no organic depository unless the matrix was present. Bone matrix has an average composition of 96 per cent collagen, 3.5 per cent polysaccharide and 0.5 per cent noncollagenous protein. Apparently it is the product of osteoblasts. The grams of hydroxyapatite (HA) per $cm.^3$ of bone tissue varies in different animal species. (Fig. 1).

Not only is the bone matrix surrounded by bone cells (osteoblasts) but it is perme-

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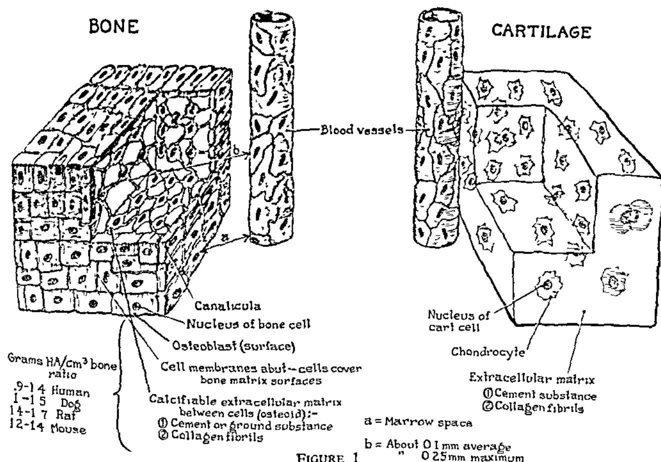


FIGURE 1

ated by bone cells (osteocytes) and their protoplasmic processes.[16] Thus there is anatomic as well as some good, though indirect, physiologic evidence, pointed out by Howard[5] and others, that the bone cells probably control what goes on in the matrix so long as they are alive. (Fig. 1)

The collagen fibrils of bone matrix are of the native 700 A variety. They are associated spatially with the earliest evidence of mineralization by apatites,[13] not only outside but inside the fibrils.[22]

Based on electron micrographs, the band regions of collagen fibrils in bone appear to be associated with the earliest evidence of mineralization. Detailed analysis of electron micrographs of embryonic avian bone suggests that tiny crystals of calcium phosphate of the apatite variety first appear near the a and the c minor collagen band regions.[4] (They have been observed in dentin on all the minor collagen bands.[20]) Still later these tiny crystals appear to establish continuity over about 500 A of the major periods between the a and the c minor bands along the collagen fibril.[20] And in fully mineralized human bone matrix from the outer rib cortex, the crystal size varied considerably with 2 peaks in the crystal length distribution curve at about 180 A and about 400 A.[19] In this last stage they are concentrated on (and probably in the fibrils) at the major or "doublet" band regions that encompass the a, b, b_1 and c minor band regions of the fibrils of newly formed bone matrix.

Although often needle shaped, these apatite crystals have been observed as tiny plaque- or tablet-shaped crystals in the outer cortex of the human rib The crystals also spread through the cement substance or polysaccharide space between the fibrils Thus bone matrix appears to go through 3 phases during mineralization uncalcified osteoid, primarily calcified osteoid and fully calcified osteoid.[16]

When the first crystal seeds are sufficient in quantity per unit volume of matrix to give an electron diffraction pattern, the pattern is that of unoriented apatite; but, i fully calcified or mature 3rd stage, the tron diffraction pattern of bone in regions where the fibrils are in parallel shows that the "C" axis or long axis (crystals and the long axis of the fibrils cide.[4,19,23]

THE APATITE CRYSTALS IN BONE MATRIX

The apatite crystals are related (fibrils of collagen in a very special wa gesting a stereochemical or template tionship between the organic collage the inorganic apatite molecules.

Collagen fibrils and the polysaccha connective tissue matrices are both hydrated.[1,21] Work over the past 5 ye given definite evidence that, as mine tion of the bone matrix occurs, the crystals displace the water[18] and n organic solids of the osteoid.[17] As cr zation proceeds, the bone matrix do shrink or expand but becomes dehy There is also some electron-microsco dence, I believe, that bone is fairly drated when fully calcified.

When a specific calcium chelator (I is used for bone decalcification, both t cium and the phosphate are re Whichever of these 2 ions was involv marily in crystal nucleation and cryst formation on the fibrils, both are l gether during EDTA decalcification.[11] tron micrographs of bone undergoin decalcification show a sharply defin between fully calcified and complet calcified collagenous bone matrix, an generalized loss of crystal density th out the decalcifying matrix This decalcification spreads centripetally fr lacunae and the canaliculi through the (EDTA) decalcifying solution pr permeates the calcified matrix If th nal water of osteoid was present, the of crystal removal would, we prop more diffuse throughout the matrix. F without contraction or expansion

specimen (when the decalcifying solution is properly buffered at about pH 7.0), the additional water content of the decalcified bone specimen, when measured closely, approximates from a volume standpoint the volume of the specimen occupied by mineral prior to decalcification.[18]

The apatite crystals not only stiffen the bones for skeletal purposes but act as a mineral depot from which mineral can be obtained by outright disbursement or heterionic exchange.

Observations by Heller-Steinberg[7] suggest that, in the first stages of bone mineral mobilization in vivo under excess parathyroid stress, the matrix may remain, but in the region about the affected cells the matrix changes its tinctorial affinities in a manner that suggests the possibility of increased matrix hydration, while the amount of mineralization may decrease to allow water increase. At any rate, the mineral became more reactive to silver stains. The interesting phenomena that she detected by light microscopy in a very narrow zone about the periphery of the most superficially located bone cells on metaphysial bone trabeculae a few hours after parathormone administration have not been studied by the electron microscope. The possibility of matrix hydration and reciprocal decalcification at an almost submicroscopic level after parathyroid hormone stimulation must eventually be studied by electron microscopy, particularly in view of recent work by Neuman and co-workers[11] that suggests that bone cells under parathyroid hormone stimulation produce citric acid, which then pours out of the bone along with calcium and phosphate. This, in turn, suggests that bone matrix decalcification during parathyroid hormone stimulation may occur preceding true "osteoclasis" if one considers "osteoclasis" as removal of bone matrix and mineral simultaneously

All other evidence that I know of obtained by light[26] and electron microscopy[2] suggests that when bone mineral is resorbed to supply large calcium and phosphate demands of the whole organism or to remodel bone, the crystals and the matrix are removed, if not simultaneously, almost simultaneously.

However, it has been recognized for some time that although the bone crystals themselves retain the fundamental apatite lattice by x-ray and electron diffraction technics, chemical analyses of bone will show variations in bone mineral due to ion substitutions on or/and in the crystals in the case of certain ions; for instance, CO_3 for PO_4, and Sr (and Na to a much lesser extent) for Ca.[12] Such an interchange by which bone may act more as a bank exchanging one ion for another depends on at least 2 factors:

1. The ability of the substituting ions to reach the crystal surfaces, which implies a water bridge for transit of such ions as strontium and Na and carbonate;

2. The permissibility of heterionic substitutions to take place on crystal surfaces often without change of the fundamental apatite lattice of the crystals themselves.

Bone crystals are exceedingly small, and, whether needles or tablets, from 25 to 50 per cent of their atoms can be calculated to be in their outermost molecular layer, since they are very thin (i.e., 25-75 A in at least 1 and often 2 dimensions). They have a surface area of 100 to 300 sq. M. per Gm., and, since they have a density of 3.0, only ⅓ $cm.^3$ of crystals weighs 1 Gm.

Also, the mineralization process, although displacing osteoid water from the matrix, reaches a state characterized as "full" calcification, which still allows in living bone a small quantity of water to remain in the matrix and on the crystal surfaces.[14]

Although in fully mineralized bone the crystal surfaces and a swarm of ions in the fluid remaining round the crystals are present, they are not nearly as available as in newly formed, incompletely mineralized bone. In the latter situation, the matrix has more water space for the movement of hydrated ions of various sizes. Thus ion sub-

stitutions would occur over short time intervals in more hydrated regions and over longer time intervals in practically all matrix regions.[24]

Bone matrix mineralization is related in some way to the collagen fibril.

As shown by Neuman[12] and others, blood is supersaturated for hydroxyapatite in respect to a normal and even a moderately low Ca × P blood product. However, Ca and P will not precipitate in blood or extravascular fluid at the normal pH of blood. Neuman demonstrated that in in vitro solutions, the Ca and the PO_4 ion concentrations must be sufficient to precipitate secondary calcium phosphate before any calcium phosphate crystals would form, including hydroxyapatite. Interestingly enough, the crystals formed in a hydrated medium are tertiary calcium phosphate or, in other words, apatite crystals, and not secondary calcium phosphate crystals even under these conditions. Apparently this is due to the fact that secondary calcium phosphate crystals spontaneously rearrange their atomic lattice to the more stable apatite arrangement when in the presence of moisture. Thus, interest has centered on the mechanism by which mineralization in bone matrix is initiated normally, since the blood is *undersaturated for crystal inception.* Apparently there is no problem in hydroxyapatite crystal maintenance (so long as the matrix remains), for the blood is *supersaturated* in relation to them. (The possibility still exists that the osteoblasts and the osteocytes can

PRIMITIVE CONCEPT OF A NUCLEATION SITE (PROBABLY AT A "BAND REGION" OF A COLLAGEN FIBRIL)

STEP I

Lysine

DNFB attaches here to the E-Amino groups

Polypeptide chain

Hydroxy Proline

(Hydrogen bond)

COLLAGEN FIBRIL (***)

ATP (*)

Ribose or Adenine

Hydroxylysine

Lysine and Hydroxylysine built into collagen fibrils (*) Larger % free to react in normally calcifying matrix

(1) Breaks this bond with water from (2)

(2) Forms this bond

(3) H Bond breaks

Phosphorylating Enzyme (*)

Inception of calcification process (**)

*Cellular products

**Occurs only if adequate phosphate in plasma to permit cell to make phosphate precursor

***Tropo collagen units join into fibrils at the cell membrane This bonding is weakened by amino-acetonitrile The "native" 700A fibril form is necessary for calcification.

FIGURE 2

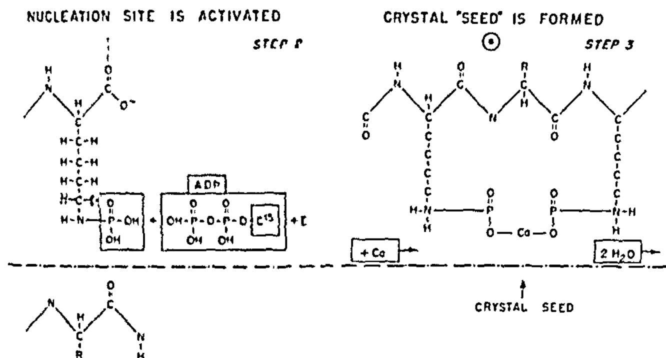


FIGURE 3

remove these crystals or partially remove them from a narrow zone of matrix surrounding the cells by locally altering their environment without removing matrix, as suggested by the parathyroid hormone experiments of Heller-Steinberg; but, if this occurs, apparently it does so only at an almost submicroscopic level of organization. Further investigation to rule out the possibility of "halisteresis" at this level must be carried out before one can be sure.)

Using reconstituted collagen fibrils of the native 700 A type as free as possible of mucopolysaccharide, Glimcher, Hodge and Schmitt[5] demonstrated that these fibrils would, by a process called heterogenous nucleation, precipitate Ca and PO_4 ions from an aqueous solution metastable in respect to secondary (or tertiary) calcium phosphate crystal formation. The biologic source of the collagen was not apparently important. The crystals formed in and on the fibrils and predominantly on the band regions of the collagen fibrils, as observed by electron microscopy.[5]

Neuman and co-workers, comparing EDTA decalcified rachitic osteoid and similarly decalcified matrix of previously normal bone in which the cells were defunct and from which all detectable calcium and phosphate had been removed, initiated hydroxyapatite crystal formation from a synthetic plasma in which the calcium and phosphate concentration was metastable in respect to crystal formation.[10]

Cartier and co-workers[3] found that they could recalcify decalcified and rachitic cartilage matrix much more easily by using ATP as the phosphorus source in their recalcifying solutions than by using inorganic phosphate. LaCroix[9] found this to be true in decalcified bone with one peculiarity: the regions in bone that before decalcification had been "fully" calcified recalcified less well than those regions that before decalcification had been slightly calcified. This suggests that once a crystal nucleation site in bone matrix is "used," it is altered somehow so that it cannot be used again.

In general, there is no clear explanation of what appears to be increased ease of bone matrix recalcification in vitro when one uses a high-energy phosphate; nor is this apparent ease of recalcification, or even recalcification in any form, proof that such in vitro calcification is the same as in vivo calcification. This work with decalcified bone matrices must eventually be reviewed by electron diffraction and electron microscopy, as Glimcher, Hodge and Schmitt have done in their experiments. Nevertheless, it does suggest that ATP may play a role in the initiation of calcification in vivo. Perhaps the bone cell produces and excretes this ATP to activate crystal nucleation sites on the collagen fibrils in the matrix that it controls.

Solomons and Irving[23] have found that collagenous matrices, such as bone and dentin that calcify normally, have in the rachitic form before calcification or when decalcified in EDTA all of their E-amino groups (which are located almost exclusively on lysine and hydroxylysine of collagen) available to DNFB (Sanger's reagent), whereas other collagen matrices only have (even after similar chemical treatment) 65 per cent or less of such groups available to DNFB. All collagenous matrices have approximately an equal number of such groups per gram of dry collagen. These E-amino groups become blocked early out of proportion to the percentage of calcification during recalcification but become available during decalcification in direct proportion to the percentage decalcification. This suggests that they may be related to the crystal nucleation sites in bone matrix.

In vivo mineralization of bone matrix is associated with collagen fibrils and apparently with nonhomogenous band regions, wherein lysine, hydroxylysine, arginine and histidine are thought to be predominantly concentrated

Polysaccharides are also present in vivo, and yet in vitro Glimcher found that polysaccharides decreased the ability of collagen fibrils to nucleate apatite crystals. Perhaps the cells must somehow in bone liberate the collagen from some close bond with polysaccharides to activate collagen nucleation sites for calcification.

The lag and spotty distribution of apatite noted in crystal formation in osteoid (in low P rickets in rats after injection of inorganic PO_4[15]) suggests to me that the bone cells that surround bone matrix must somehow, under the conditions of the rickets model used, prepare the phosphate for matrix deposition and/or open up nucleation sites for crystal seed formation on the collagen fibrils. The time lag and spotty distribution of the initial apatite deposition in the first stages of calcification of rachitic osteoid suggest further that matrix mineralization is not a simple chemical union between diffusible PO_4 ions in blood and some readily available phosphate receptor on collagen fibrils in the osteoid.

Some observations concerning the calcification of bone matrix have been presented, but the exact molecular mechanism by which bone matrix calcification takes place is still obscure. A very vague guess about this mechanism is outlined in Figures 2 and 3.

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Le Relationes Inter Crystallos, Collageno, e Aqua in le Matrice de Osso

Summario in Interlingua

In recente tempores, numerose investigatores ha studiate le osso per medio de moderne technicas histologic, physiologic, biochimic, e biophysic. Un facto pare esser establite in ultra de omne dubita: Le inorganic crystallos de phosphato de calcium e

le fibrillas de collageno in le matrice ossee es intimemente associate. Super le base del currentemente cognoscite factos, un prime approximation del relation inter le crystallos e le collageno del matrice de osso al nivello molecular es delineate diagrammaticamente. Tamen, le autor signala que le lacunas in nostre informationes relative al mechanismo del mineralisation in le matrice de osso es si extense que le hic-proponite relation inter crystallos e collageno es necessarimente multo primitive e representa, in le caso le plus favorabile, non plus que un approximation provisori del factos real.

9

Hyaline Cartilage; The Histochemical Characterization of the Extracellular and the Intracellular Compartments*

LILLIAN EICHELBERGER, PH.D.†

During the past quarter of a century important advancement has been made in determining the distribution of water and electrolytes in the soft tissues of the body: skeletal muscle, liver, kidney, brain, skin, heart, etc. Both the type and the concentration of the ions that occur within and outside the cells of the soft tissues have been studied intensively by numerous able investigators. Over the same period, however, little attention has been given to the hard connective tissue—hyaline cartilage—the predominant and most typical cartilage of the body. In adult mammals it is to be found in the respiratory passages, in the ventral ends of the ribs and on the surfaces of bones within the joints.

In 1954 Manery[19] surveyed the literature on the chemical analyses of cartilage from the earliest analyses up to that time. Since then additional work on cartilage has been carried on in our laboratories, not only to study the tissue as a whole, but also to establish the chemical estimation of the proportion of the mass of the extracellular and the intracellular compartments. The present discussion will be limited to mammalian hyaline cartilage as a tissue rather than its development, function, etc., and summarizes our recent studies in which attempts have been made to bridge some of the gaps between the chemical and the morphologic approaches to the study of this tissue. The whole course of science, as we understand it, is a process of providing just such refinements of thought on specified areas that will permit distinctions to be made more consistently than was possible previously.

In the highly organized tissue of cartilage, several different types of cells exist, and at present these cannot be separated mechanically for chemical analyses. Therefore, the water and electrolyte content of all the cells and their outside environment must be deduced from analyses of the whole tissue. At the outset it is necessary to state the general properties covering the tissue cartilage and to enumerate the assumptions concerning this tissue that are pertinent to this presentation.

Briefly, cartilage is a specialized form of connective tissue composed of chondrocytes

* The work summarized here was done in collaboration with Doctors T. D Brower, J. S. Miles, W. H. Akeson and Michael Roma. The investigations were aided by grants from the National Institute of Health (Grant A-972 from the National Institute of Arthritis and Metabolic Diseases) and grants from The Home for Destitute Crippled Children of the University of Chicago.

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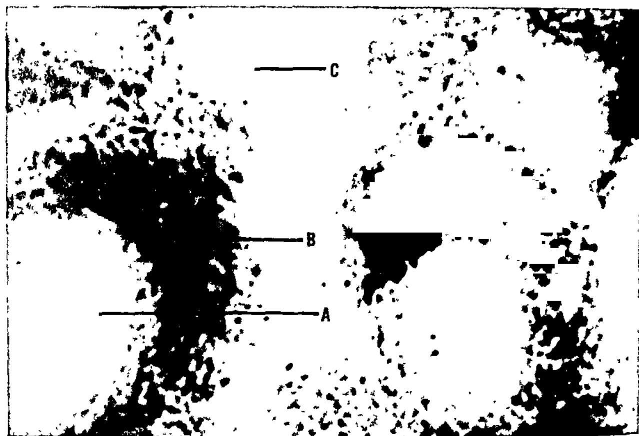


Fig. 1. Photomicrograph of a section of femoral articular cartilage. The section was stained to show the distribution of chloride, which appears as minute yellow granules (black granules in the photomicrograph). The cells are free of granules. (A) The cells; (B) granules representing chloride; and (C) the matrix. (Brower, T. D.: J. Bone & Joint Surg. 38-A:656)

in an intercellular substance.[23] The cartilage cells or the chondrocytes are the intracellular phase and consist of water and solids. The intercellular substance has a fibrillar structure, the fibers being mainly of collagen (a protein or a mixture of proteins). Between the fibers is a fluid similar in composition to other extracellular fluids and an amorphous ground substance characterized by a mucopolysaccharide—chondroitin sulfate.[15] Thus the extracellular phase of cartilage consists of 3 subphases: (a) the connective tissue phase containing the fiber solids and some extracellular fluid; (b) the chondroitin sulfate phase, which is in the spaces between the collagen fibers; and (c) the extracellular fluid, which is an ultrafiltrate in the interstices The tissue fluid and the ground substance are considered to be interconnected throughout [5,14]

The assumptions concerning cartilage that are pertinent here are:

1. The connective tissue of cartilage resembles all over connective tissues in water, nitrogen and electrolyte concentrations. As a consequence, we have judged that the connective tissue of cartilage resembles that of the dense connective tissue—tendon—and that the collagen nitrogen of cartilage exemplifies the total connective tissue weight in cartilage. This concept was based on the work of Manery, Danielson and Hastings.[20] Also, Partridge[26] has stated that the ground substance of cartilage is similar to that occurring in loose connective tissue.

2. The amounts of water, chloride, sodium and potassium identified with the connective tissue of cartilage correspond to those found in the dense connective tissue—tendon.

3. Under normal metabolic conditions the chondrocytes of cartilage contain no chloride. This concept has been clarified by the microscopic work of Brower.[3] In 1956, Brower, using a silver nitrate stain for chloride, as described by Gersh,[14] showed by qualitative microscopic method that the dense collection of silver oxide granules was in the extracellular matrix immediately adjacent to the chondrocyte. No granules were seen within the cells. In his prepared cartilage sections the chloride was precipitated as silver chloride and then converted by radiation with an arc lamp into silver oxide, which is seen as yellow brown granules. A photomicrograph of a section of femoral cartilage as shown by Brower is depicted in Figure 1.

Later work by Brower[4] on epiphysial cartilage showed again that the location of the silver oxide granules (chloride stain) was in the extracellular matrix and not within the chondrocytes (Fig. 2).

Knowledge of the mass of the extracellular and the intracellular compartments of hyaline cartilage and the concentrations of constituents within these compartments provides information of physiologic importance. First, the patterns for normal cartilage must be known in order that comparisons can be made with the patterns of cartilage obtained under different experimental or pathologic conditions. For example, what has happened to the 2 phases or compartments of the tissue? What is the concentration of the connective tissue fibers and chondroitin sulfate in the extracellular phase? And, of functional importance, what is the water content of the chondrocytes? These are some of the questions that can be answered from the histochemical characterization of this tissue.



FIG 2. Photomicrograph of a section of epiphysial plate cartilage The cells are free of granules. The entire matrix stains heavily and evenly for chloride. (Brower, T. D.: J. Bone & Joint Surg. 41-A:1518)

The histochemical characterization of hyaline cartilage will be presented. The data have been grouped into 2 divisions: (1) those concerned with the normal characterization of articular cartilage; and (2) those concerned with the characterization of costal cartilage.

SYMBOLS AND METHODS OF CALCULATION

In addition to the usual chemical symbols, the accompanying notations and methods of calculation are employed in this discussion. The weight of the connective tissue solids in 100 Gm. of cartilage solids was estimated from the collagen nitrogen values by the method of Manery, Danielson and Hastings.[20] Also, the amounts of water, chloride, sodium, potassium, calcium and magnesium identified with the estimated amount of connective tissue solids were calculated as shown below. The assembled values for the water, nitrogen and electrolyte content of dog tendon were taken from papers on the chemical analyses of tendon[10,27,29] and are as follows:

100 Gm. of tendon solids is associated with 157 Gm. of water, 17.2 Gm. of total nitrogen, 15.8 Gm. of collagen nitrogen, 20.65 mEq. of chloride, 20.2 mEq. of sodium, 3.4 mEq. of potassium, 1.8 mEq. of calcium and 0.56 mEq of magnesium.

Parentheses ( ) represent mEq. or Gm. per Kg. of tissue.

Brackets [ ] represent mEq. or Gm. per Kg. of tissue water.

Braces { } represent mEq. or Gm. per Kg. of phase.

Subscript small s refers to 100 Gm. of cartilage solids.

Subscript large S refers to 1 Kg. of fresh cartilage.

$(F)_s$ = Gm. connective tissue solids in 100 Gm. of cartilage solids.

$(F)_s = 100 \times (\text{collagen nitrogen})_s/15.8$.

mEq. chloride associated with $(F)_s = 20.65 \times (F)_s/100$.

mEq. sodium associated with $(F)_s = 20.2 \times (F)_s/100$.

mEq. potassium associated with $(F)_s = 3.4 \times (F)_s/100$.

Δ denotes amount of constituent not associated with the connective tissue solids which = total amount in 100 Gm. cartilage solids —amount associated with $(F)_s$.

CSA denotes chondroitin sulfate. The mass of chondroitin sulfate per 100 Gm. of cartilage solids was calculated from the mM. of sulfate obtained after hydrolysis of the cartilage solids, using the molar weight of chondroitin sulfuric acid to be that derived by Levene.[17]

$(E)_s$ = Gm. of extracellular solids/100 Gm. cartilage solids $= (F)_s + CSA_s + \Delta Na_s$.

$(C)_s$ = Gm. of intracellular solids/100 Gm. cartilage solids $= 100 - (E)_s$.

ΔNa_s = total sodium content in 100 Gm cartilage solids—amount of sodium associated with $(F)_s$.

$(E)_S$ = Gm. extracellular solids/Kg. of fresh cartilage.

$(C)_S$ = Gm. intracellular solids/Kg. of fresh cartilage.

$(F)_S$ = Gm. connective tissue solids/Kg fresh cartilage $= (F)_s \times$ total solids/100

$(H_2O)_F$ = Gm. water associated with $(F)_S$ $= 157 \times (F)_S/100$.

$(Cl)_F$ = mEq. chloride associated with $(F)_S = 20.65 \times (F)_S/100$.

$(Cl)_S$ = mEq. chloride per Kg. of fresh cartilage $= (Cl)_s \times$ total solids/100.

$\Delta Cl_S = (Cl)_S - (Cl)_F$.

U denotes ultrafiltrate.

Cl_U = mEq. chloride/Kg. serum water $\times$ 0.0099/0.0095.

$(H_2O)_U$ = Gm. of ultrafiltrate water $= \Delta Cl_S \times 1000/Cl_U$.

$(H_2O)_E$ = Gm. extracellular water/Kg. cartilage $= (H_2O)_F + (H_2O)_U$.

$(E)_S$ = Gm. solids in extracellular compartment $= (F)_S + CSA_S + \Delta Na_S$.

$(E)_T$ = total Gm. of extracellular compartment per Kg. cartilage $= (H_2O)_E + (E)_S$.

$(C)_T$ = total Gm. of intracellular phase per Kg. of cartilage $= 1000 - (E)_T$.

$(H_2O)_C$ = Gm. intracellular water per Kg. cartilage = total water $- (H_2O)_E$

$(C)_s$ = total solids − $(E)_s$ = Gm. solids in intracellular compartment.

$[H_2O]_C$ = Gm. water per Kg. of chondrocytes = $(H_2O)_c \times 1000/(H_2O)_c + (C)_s$.

Equations used for the calculation of the distribution of cations. Potassium used for example:

$[K]_F$ = mEq. of K per Kg. of extracellular fluid = mEq. K per Kg. of serum water × 0.95.

$(K)_T$ = mEq. per Kg. of fresh cartilage = mEq. $(K)_s$ × total solids/100.

In $(H_2O)_F$ = mEq. K in ultrafiltrate = $[K]_F \times (H_2O)_t/1000$.

Associated with $(F)_s$ = mEq. K associated with $(F)_s = 3.4 \times (F)_s/100$.

In (C) = mEq. K in chondrocytes = $(K)_T$ − (In $(H_2O)_E$ + assoc. with $(F)_s$).

$(K)_c$ = mEq. per Kg. of chondrocytes = $(K)_c \times 1000/(H_2O)_C + (C)_s$.

$[K]_c$ = mEq. K per Kg. of chondrocyte water = $(K)_c \times 1000/(H_2O)_c$.

Puppies from which cartilage specimens were obtained were bred in our laboratories. At a specified age the animals were killed to obtain cartilage and samples of blood from each animal. The articular cartilage was shaved in thin pieces from the articular sur-

TABLE 1.* MEAN VALUES FOR ARTICULAR CARTILAGE FROM PUPPIES' ORIGINAL DATA

	H_2O Gm.	Cl mEq	Na mEq	K mEq.	Ca mEq.	Mg mEq.	Total N Gm.	Collagen N Gm.	CSA Gm.
Group 1: 6-12 weeks of age (5)†									
Serum	951	116	145	4.64	6.2	2.32	7.86		
Cartilage									
Front	801	32.2	102	11.7	14.1	6.23	12.6	8.28	18.5
Hind	800	32.3	108	11.7	13.2	5.90	12.6	8.17	19.8
Group 2: 13-14 weeks of age (6)									
Serum	936	112	146	4.74	5.63	2.22	7.74		
Cartilage									
Front	779	29.5	96	10.7	19.6	6.18	13.3	9.11	16.1
Hind	783	30.7	102	10.4	16.7	5.67	13.4	8.90	16.4
Group 3: 15-16 weeks of age (6)									
Serum	928	111	146	5.01	5.80	2.08	8.92		
Cartilage									
Front	773	30.1	86.8	9.28	14.4	5.22	13.9	9.16	15.7
Hind	774	30.8	90.6	9.05	13.7	4.70	13.6	9.27	15.3
Group 4: 17-20 weeks of age (8)									
Serum	930	110	147	5.06	5.68	1.91	8.48		
Cartilage									
Front	754	27.2	78.3	8.55	20.1	3.91	13.7	9.18	12.3
Hind	766	30.7	79.3	9.09	23.2	4.60	13.5	8.89	11.7
Group 5: 21-25 weeks of age (6)									
Serum	927	110	143	4.87	5.60	2.39	8.54		
Cartilage									
Combined . . .	748	27.5	76.7	8.37	34.9		13.5	9.59	9.1

* Serum values are given in units per Kg. of serum Cartilage values are given in units per 100 Gm. of cartilage solids, except water values, which are given in Gm per Kg. of fresh cartilage.

† Figures in parentheses indicate number of animals

(Eichelberger, Akeson & Roma: J Bone & Joint Surg. 40-A:144)

faces at the instant the joint was opened. For the costal cartilage the ventral ends of the ribs were excised. Complete procedures and methods for chemical analyses have been given in detail in previous papers.[1,7,8,9,11,12]

NORMAL ARTICULAR CARTILAGE

The observed data for normal articular cartilage as removed from the front and the hind legs of puppies varying in age from 6 weeks to 25 weeks of age are given in Table 1. The derived data are shown in Figures 3 to 5. In Figure 3 are shown the estimated weights of extracellular (E), and intracellular solids (C), in 100 Gm. of total cartilage solids. The percentage of the chondroitin sulfate solids is illustrated by the black areas; the percentage of the connective tissue solids, by the unshaded areas; and the percentage of the chondrocyte solids, by the dotted areas. The salient point is that in this tissue the weight of the solids in the extracellular compartment is more than twice the weight of the cell solids. Therefore, when a weight of total cartilage tissue is being considered, the greater part of the solid mass is extracellular mass and not cell solid mass. This is the opposite of what is found in the soft tissues.

The concentration of connective tissue fibers and chondroitin sulfate in the extracellular compartment must be an important factor in the transport of materials to and from the chondrocytes, and it is interesting to show the percentage of connective tissue solids and chondroitin sulfate solids in 100 Gm. of extracellular solids. Figure 4 is a representation of connective tissue solids, illustrated by the clear areas, with the chondroitin sulfate solids, illustrated by the black areas, in 100 Gm. of extracellular solids. At a glance, the percentage of connective tissue solids makes up around 75 per cent of the total extracellular solids, while the chondroitin sulfate represents 25 per cent of the extracellular mass. Chondroitin sulfuric acid is a long-chain polysaccharide consisting of residues of glucuronic acid and acetylgalactose-amine-sulfuric acid and, according to Kurt H. Meyer and his associates,[21] carboxyl and SO_3H groups. Boyd and Neuman,[2] Neuman, Boyd and Feldman,[25] and recently

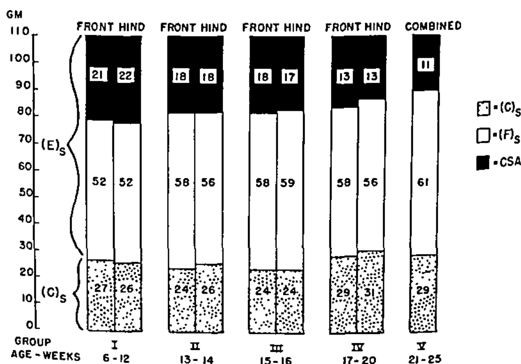


FIG 3 Percentage distribution of solids in 100 Gm. of articular cartilage solids

Mathews[22] and Howell *et al.*[16] have shown that the chondroitin sulfuric acid is a principal cation-binding constituent of cartilage. Therefore, the large amounts of sodium present in cartilage must be available to form salts with the chondroitin sulfuric acid and not with the connective tissue proteins. Manery, Danielson and Hastings[20] have shown that the tendon proteins exist in a form that is not base binding. Late work of Mathews shows that sodium chondroitin sulfate is present in cartilage in the form of a non-collagenous protein complex.[21] Therefore, it is necessary for the weight of the connective tissue solids (collagenous) and the chondroitin sulfate solids (noncollagenous) to be expressed separately.

From the original data and the above description of the amounts of extracellular and cellular solids in 100 Gm. of total solids, the mass of the extracellular and the intracellular compartments per Kg. of wet total cartilage can readily be obtained. In determining these values for wet cartilage from the 100 Gm. of

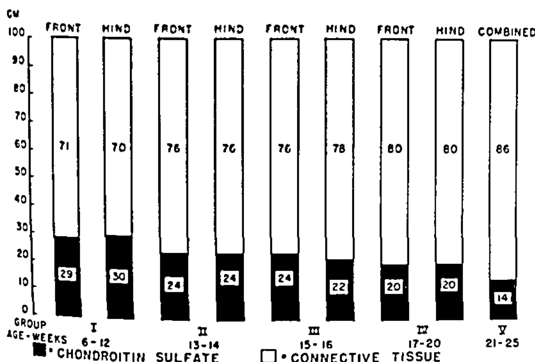


Fig. 4. Percentage distribution of solids in 100 Gm of extracellular solids of articular cartilage.

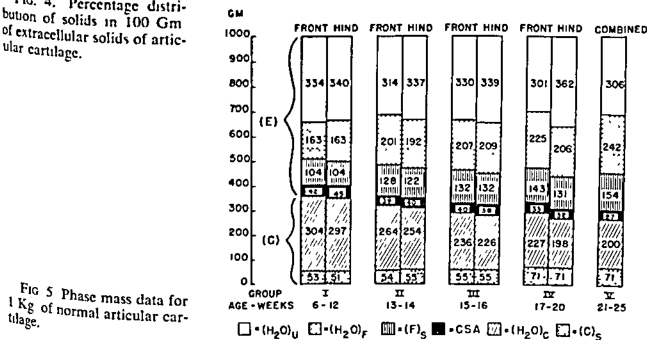


Fig 5 Phase mass data for 1 Kg of normal articular cartilage.

dry cartilage solids, the analytic data for the total water and total solids per Kg. of cartilage must be known. Figure 5 depicts the phase mass data for 1 Kg. of articular cartilage from the 5 groups of puppies. Graphic representation of the extracellular compartment consists of (1) ultrafiltrate water illustrated by clear areas; (2) the connec tissue phase consisting of the connec tissue solids, illustrated by the perpendic cross-hatched areas, and the associa water, illustrated by the double cr hatched areas; and (3) the chondroitin fate phase illustrated by the black area.

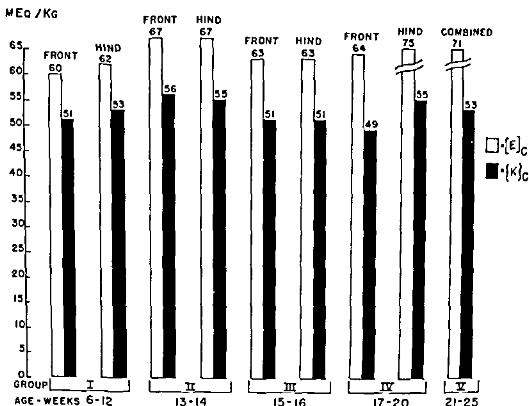


Fig. 6. Intracellular potassium concentration.

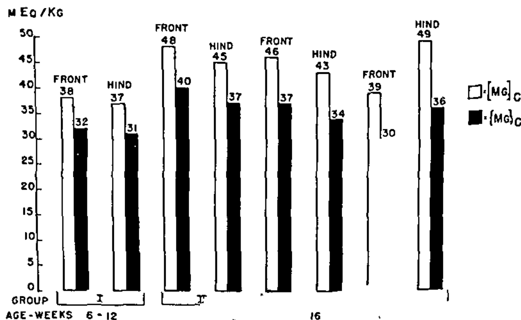


Fig. 7. Intracellular

intracellular compartment consists of the intracellular water, illustrated by the single diagonally hatched areas, and the intracellular solids, by the dotted areas.

It is well known, as emphasized by Lowry and Hastings[18] in 1942, that there is an optimal relation between the mass of extracellular and intracellular phases, and deviation from the optimum in either direction may result in a disturbance in the metabolic activity of the cells.

The mass of the compartments in cartilage now being known, the intracellular concentration of many constituents can be derived. Figures 6 and 7 show the estimated concentration of potassium and magnesium, respectively, expressed per Kg. of chondrocytes and also per Kg. of chondrocyte water in both the cartilage from front and hind legs. After the total content of each constituent, $(K)_T$ and $(Mg)_T$, was corrected for the amount in the extracellular phase (E), i.e., the amount in the ultrafiltrate, $(In\ (H_2O)_{U})$, plus the amount associated with the connective tissue fibers (Assoc. with $(F)_R$), the remaining portion was assigned to the tissue cells (C).

TABLE 2. DISTRIBUTION OF SODIUM BETWEEN SERUM AND ARTICULAR CARTILAGE

			Extracellular Phase			
	$[Na]_F$ (mEq.)	$(Na)_T$ (mEq.)	$In\ (H_2O)_U$ (mEq.)	$+$ Assoc. with $(F)_R$ (mEq.)	ΔNa (mEq.)	CSA_R (mM.)
Group 1						
Serum	144.5					
Cartilage						
Front		204.8	48.3	21.0	135.5	78.6
Hind		217.0	49.1	21.0	146.9	79.0
Group 2						
Serum	148.2					
Cartilage						
Front		212.4	46.5	25.8	140.1	75.9
Hind		222.2	49.9	24.6	147.7	76.0
Group 3						
Serum	149.1					
Cartilage						
Front		196.9	49.2	26.7	121.0	76.6
Hind		203.9	50.5	26.9	127.6	73.6
Group 4						
Serum	150.0					
Cartilage						
Front		192.9	45.4	28.9	118.6	64.7
Hind		185.4	54 3	26.5	104.5	58 4
Group 5						
Serum	146.0					
Cartilage						
Combined		193.4	44.7	31 1	117 6	48.9

$[Na]_F$ = milliequivalents Na per Kg extracellular fluid = milliequivalents Na per Kg. serum water × 0 95.
$(Na)_T$ = milliequivalents Na per Kg fresh cartilage.
$In\ (H_2O)_U$ = milliequivalents Na in ultrafiltrate = $Na_F \times (H_2O)_U/1{,}000$.
Assoc with $(F)_R$ = milliequivalents Na associated with $(F)_R = 20\ 2 \times (F)_R/100$.
ΔNa = total determined sodium − Na in extracellular phase = $(Na)_T - [In\ (H_2O)_U +$ Associated with $(F)_R]$
CSA_R = mM. chondroitin sulfate per Kg. of fresh cartilage
(Eichelberger, Akeson & Roma: J. Bone & Joint Surg. 40-A:150)

From this value the concentrations were expressed per Kg. of chondrocytes, $\{K\}_C$ and $\{Mg\}_C$, and also per Kg. of chondrocyte water, $[K]_C$ and $[Mg]_C$. The concentration of magnesium in cartilage cells is greater than that of soft tissue cells, while the concentration of potassium is less than half that of the soft tissue cells. Therefore, it seems that some other cation is present in the chondrocyte.

The distribution of sodium must be considered. Table 2 gives some of the distribution values. The ΔNa values, by speculation, represent the amount of sodium associated with the chondroitin sulfuric acid. From the values of ΔNa and the values for the chondroitin sulfate given in the last column of the table, it will be noted that, when the chondroitin sulfate values are high, the ΔNa values are high, and conversely. Mathews[22] has shown that the binding sites of acid mucopolysaccharides depend largely on the electrostatic interactions between neighboring charged groups on the polyion.

NORMAL COSTAL CARTILAGE

Costal cartilage, unlike the naked articular cartilage, is covered by a layer of dense connective tissue (perichondrium), which has to be removed by stripping before chemical analyses are made.[12]

NORMAL VALUES

Mean observed values for costal cartilage from puppies are given in Table 3 along with the mean values for serum constituents for each group. Five groups of puppies were included and arranged according to age.

The calcium content per 100 Gm. of cartilage solids is found to be of a constant value up to 13 weeks of age: 10.98 mEq. in the 3- to 7-week-old puppies and 11.0 mEq. in the 8- to 12-week-old puppies. Beyond these ages the calcium content increased, indicating the formation of extra calcium salts

TABLE 3.* MEAN VALUES FOR COSTAL CARTILAGE FROM PUPPIES' ORIGINAL DATA

	H_2O Gm.	*Cl* mEq	Na mEq.	K mEq.	*Ca* mEq.	*Mg* mEq.	Total N Gm.	Collagen N Gm.	SO_4 mM	P mM.	CO_2 mM.
Group 1. 3-7 weeks of age											
Serum	937	108 1	141.5	5.62	5.58	2.20	7.24				
Cartilage .	781	22.50	103.4	25.25	10.98	6.89	11.16	6.83	47.2	5.86	0.80
Group 2. 8-12 weeks of age											
Serum .	935	108.8	140.5	5.32	5 40	2.46	7.73				
Cartilage . . .	760	21.84	97 5	21.21	11 03	7.05	12.03	7.95	46.1	5.51	0.70
Group 3. 13-16 weeks of age											
Serum . .	931	111 7	144.2	5.05	5.33	2.22	8.56				
Cartilage . . .	748	20.37	99.6	11 10	25.17	7.20	12.08	7.55	49.0	6 60	1 05
Group 4 17-19 weeks of age											
Serum . . .	930	110 5	146 5	5.12	5.36	2 09	8.63				
Cartilage . . .	732	16 80	97 1	9.60	127.5	9.13	11.25	7 40	47.0	32 62	6.17
Group 5. 22-30 weeks of age											
Serum . . .	929	109 6	143.6	4.90	5.53	2.40	8.90				
Cartilage . .	700	12 98	83.5	11.50	272.0	11.30	9 47	6.10	43 4	107.2	18.0

* Serum values are given in units per Kg of serum. Cartilage values are given in units per 100 Gm of cartilage solids, except total water, which is given in Gm. per Kg of fresh tissue.
(Eichelberger & Roma. Am J Physiol 178:298)

in the tissue. Consequently, to study costal cartilage before the formation of the additional calcium salts, the cartilage must be taken within the first 12 weeks of life of the puppy. In Group 3, 13 to 16 weeks of age, the calcium content per 100 Gm. of cartilage solids was 28.2 mEq. If 12 mEq. of the calcium is identified with the original 100 Gm. of cartilage solids, 13 mEq. of the total amount remains to be allocated to the additional calcium salt formation in the cartilage of this age group. In Group 4, 17 to 19 weeks of age, the total calcium per 100 Gm. of solids was 127 mEq. After deducting 12 mEq. of calcium identified with the young cartilage solids, there remains 115 mEq. that has been formed in this age group. Likewise, in Group 5, 22 to 30 weeks of age, the total calcium content of 272 mEq. less the 12 mEq. leaves 260 mEq. of calcium that has been formed during 30 weeks of life. From these corrected calcium values the bone salts were calculated as follows:

If bone salts are considered to be hydroxyl apatite $(Ca_3(PO_4))_3Ca(OH)_2$,[6] and if 12 mEq. of calcium per 100 Gm. of cartilage solids represents the calcium concentration in the tissue before any calcification takes place, the weight of the extra calcium salt formation can be calculated, the total calcium content in mEq. — 12 equaling the calcium of the formed bone salt. Using the hydroxyl apatite as the formula for bone salts, 20 mEq. of calcium represents 1 mEq. of bone salt or 1.004 Gm. of hydroxyl apatite. To summarize: mEq. Ca of bone salt formed = total mEq. Ca — 12; 20 mEq. Ca equivalent of 1 mEq. bone salt or 1.004 Gm. of bone salt; mass of bone salts in grams = mEq. Ca of bone salt × 1.004/20.

From the excess calcium values the weights of the bone salts were calculated. In Group 3 a mean of 0.65 Gm. of bone salt was deposited; in Group 4, a mean of 5.8 Gm.; and in Group 5, a mean of 12.5 Gm. Along with the increases in the bone salts there occurred increases in the total phosphorus and the total CO_2. For example, in

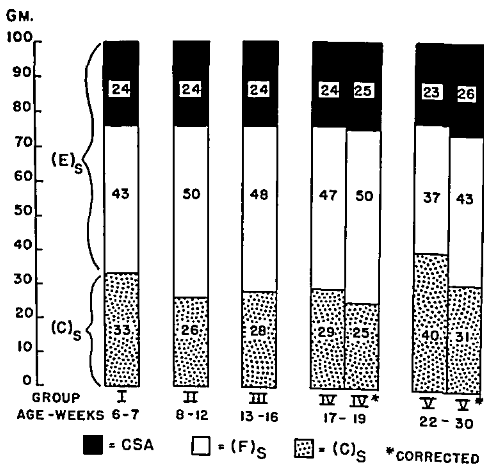


Fig. 8. Percentage of extracellular and intracellular solids in 100 Gm of costal cartilage solids (Eichelberger & Roma. Am. J Physiol 178:300)

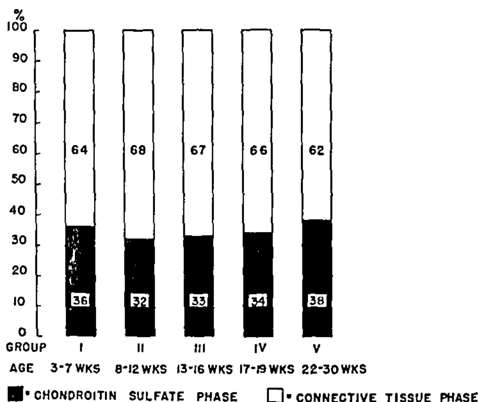


FIG. 9. Percentage distribution of solids in 100 Gm of extracellular solids. (Eichelberger & Roma: Am J. Physiol. 178:300)

Groups 1 and 2, in which no extra calcium was formed, the average total P was 5.8 and 5.5 mM., respectively, while in Groups 4 and 5, in which 115 and 260 mEq. of extra calcium had been formed, the total P values were 32.6 and 107.2 mM., respectively.

The derived data for the estimation of the extracellular and the intracellular solids per 100 Gm. of total cartilage solids are given in Figure 8. In the pairs of columns representing Groups 4 and 5, the first columns are the weights given without a correction for the calcification, while the adjoining columns illustrate the weights after corrections for the weight of the formed bone salts. If the "corrected" data (bone salt-free basis) of Groups 4 and 5 are compared with the younger Groups—1, 2 and 3—in which no correction had to be made, the points of interest are the mass of the chondrocytes is larger in the youngest group, and the mass of the chondroitin sulfate phase increased in Groups 4 and 5. When the percentage distribution of solids in the extracellular phase is considered—Figure 9—the largest chondroitin sulfate percentage is found in the youngest puppy group and in the puppies in which bone salts are being formed. Therefore, costal cartilage tissue does not seem to decrease in chondroitin sulfate concentration as the animal increases in age but increases as the puppies increase in age, at least up to 30 weeks, or at the time that bone salts are being made.

Using the corrected values for costal cartilage solids, the histochemical patterns of the cartilage can be calculated easily. The extracellular compartment, consisting of the chondroitin sulfate phase plus the connective tissue solids with their associated water and ultrafiltrate, and the intracellular compartment, consisting of solids and water, are shown graphically in Figure 10. The first 2 groups of puppies in which there had been no formation or deposition of bone salts may be designated as the control patterns for costal cartilage. The groups 3, in which there has been little bone salt formation, and 4 and 5, in which there has been much bone salt deposition, may be used to portray the effects of calcification.

When the control patterns are compared with the calcification patterns, the greatest changes are found in the extracellular com-

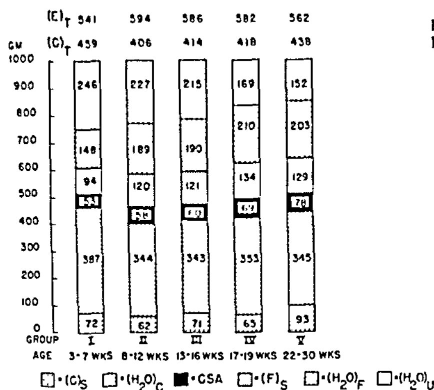


FIG. 10. Phase mass data for 1 Kg. of cartilage. (Eichelberger & Roma: Am. J. Physiol. 178:303)

partments. Both the weight of the fiber solids and the mass of the chondroitin sulfate increased in Groups 4 and 5. Therefore, it seems that at the time of bone salt formation there is an increase in the chondroitin sulfuric acid concentration. At the time of the increase in the chondroitin sulfuric acid concentration there was a decrease in the volume of interstitial fluid. Some of this decrease in the interstitial fluid volume could result from the increase in the mass of the chondrocytes in the older puppies.

The total calcium concentration per Kg. of fresh cartilage must be considered. Before calcification starts, the average calcium per Kg. of tissue was 24.1 mEq. in Group 1 and 26.0 mEq. per Kg. in Group 2. In Group 1, after the total amount of calcium, 24 1 mEq , was corrected for the extracellular Ca^{++} in the 246 Gm. of ultrafiltrate (0.80 mEq.) and the amount of Ca associated with the 94 Gm. of connective tissue solids (1 7 mEq), there remained 21 6 mEq to be allocated to some extracellular substance, probably chondroitin sulfuric acid. Likewise in Group 2, the extracellular Ca^{++} in 227 Gm of $(H_2O)_U$ was 0.79 mEq., and the calcium associated with 120 Gm. connective tissue solids was 2.16 mEq., leaving 23.0 mEq. One of the most important roles of chondroitin sulfuric acid may be that of accumulation of calcium by the acid groups.

In the intracellular compartment, consisting of solids of the chondrocytes and their water, the largest mass of chondrocytes was found in the oldest age group. In all groups, the percentage of intracellular water was quite constant, indicating that the architecture of the chondrocytes had not changed over the life period of 30 weeks for puppies. In Group 1, the percentage of intracellular water was found to be 84 per cent; in Group 2, 85 per cent; in Group 3, 83 per cent; in Group 4, in which calcification had not progressed very far, 84 per cent; and in Group 5, in which calcification had progressed extensively, 79 per cent.

CONCLUSION

Cartilage, a hard tissue, can be considered to be composed of 2 compartments, extracellular and intracellular. The mass of each compartment can be derived, as well as the

composition of the 2 compartments. The extracellular compartment consists of 3 subphases: the connective tissue phase, the chondroitin sulfate phase and the fluid in the interstices. The cellular compartment, or the chondrocytes, consists of solids and water. The evaluations for normal articular and costal cartilage have been presented in the hope that future work will be done on the quantitative alterations occurring with disease in this tissue.

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Cartilagine Hyalin: Le Characterisation Histochimic de Aqua, del Electrolytos, de Tissu Conjunctive, e de Sulfato de Chondroitina in le Compartimentos Extracellular e Intracellular

Summario in Interlingua

Cartilagine hyalin, articular e costal, ha essite describite como un forma special de tissu conjunctive, componite de chondrocytos in un substantia intercellular. Proque a iste tempore il non es possibile effectuar un separation mechanic del cellulas de iste altemente organisate typo de tissu ab le elementos adjacente, le phases histologic del tissu, tanto extra- como etiam intracellular, esseva derivate con le uso de un deductive methodologia chimic quantitative. Es presentate le resultante characterisation histochimic de cartilagine tanto articular como etiam costal. In un specimen de cartilagine fresc, le massa chondrocytic es le phase intracellular que es componite de un massa de aqua e un massa de solidos. Le substantia intercellular ha un structura fibrillar; le fibras consiste principalmente de collageno, e inter le fibras se trova un liquido (simile in composition a altere liquidos extracellular) e un amorphe substantia fundamental que es characterisate per le mucopolysaccharido sulfato de chondroitina. Assi le compartimento extracellular pote esser describite per un lista de tres sub-phases: (1) le massa del phase de tissu conjunctive continente le solidos de fibra e un certe quantitate de liquido extracellular; (2) le massa del phase de sulfato de chondroitina; e (3) le massa del liquido extracellular in le interstitios. Es presentate normas del massa de cata un del phases que es presente in un kilogramma de cartilagine fresc, tanto articular como etiam costal. Iste normas identificatori pote servir como approximationes pro le studio de alterationes quantitative in le massas relative del phases e etiam del composition del phases que resulta de processos pathologic.

10

Histochemical Changes in the Early Stages of Calcification

J. T. Irving*

Within recent years acid mucopolysaccharides have been implicated increasingly in the process of calcification,[3,5,15] but it has been difficult to understand why cartilage, with a high content of chondroitin sulfate, does not normally calcify. In this chapter the author describes a staining method, using Sudan black B, that would appear to be specific for a substance, probably of acid mucopolysaccharide nature, found only at sites in teeth and in bone where calcification is being initiated.

It is known that Sudan black will stain some acid mucopolysaccharides; for example, the granules of mast cells,[12,21] though this stain is employed more commonly for visualizing lipid material. However, when tissues were treated with pyridine, alcohol or benzol before decalcification, it was found that Sudan black then stained in a specific and characteristic manner. The results with teeth, which parallel those for bone, have already been reported.[9]

METHODS AND MATERIALS

Material. Normal rats of the Wistar Institute strain were used, fetal, young and adult animals being employed. Some experiments were conducted with rachitic animals. The rats were killed with coal gas or intraperitoneal Nembutal In addition, human fetal material, approximately 3 months of age, was studied.

Histologic Methods. Rats. The upper ends of the tibias and the bones round the upper incisor teeth were examined. The tibias were cleaned of most of the adherent muscle and were fixed in formol-saline, Baker's formol-calcium solution[1] or absolute alcohol. Then they were treated by one of the following procedures:

1. Decalcified in either 5 per cent nitric acid-formol mixture or in 0.5 M. ethylenediaminetetraacetic acid, divided longitudinally and washed, embedded in gelatin and cut in the longitudinal plane on the freezing microtome;

2. Placed in pyridine for 2 hours at room temperature, with a change at 1 hour. Then placed in pyridine at 60° C. for 24 hours, washed, decalcified and mounted as described under 1;

3. Placed in absolute alcohol at 60° C. for 24 hours, washed and treated as under 1;

4. Placed in absolute alcohol for 24 hours and then in benzol at 60° C. for 24 hours, brought back to alcohol, washed and treated as described in 1.

The skulls were removed, skinned and fixed in one of the fixatives mentioned above. Then they were split longitudinally, and the brain, the eyes and bone behind the molar teeth were removed. After that one of the four procedures was adopted, and the sections were cut in the longitudinal plane of the incisor teeth.

Human Fetal Material. This was obtained primarily for a study of the teeth, but the mandible and the alveolar bones were

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also available for study. The mandibles were removed and fixed in formol-saline, treated with pyridine as described above, decalcified with nitric acid-formol mixture, cut into small pieces and, after being embedded in gelatin, cut transversely on the freezing microtome.

Staining Methods. SUDAN BLACK B. This stain was obtained from George T. Gurr, London. The method described by Baker[2] was employed but without counterstaining. The gelatin-embedded sections were put into 70 per cent alcohol for a few seconds and then were stained for 2 to 3 minutes in a saturated solution of Sudan black in 70 per cent alcohol. After a quick rinse in 70 per cent alcohol, they were placed in 50 per cent alcohol for 1 minute, washed in several changes of distilled water and mounted in glycerin jelly. Attempts were made to dehydrate the sections and mount them in balsam, but, unless this was done quickly, most or all of the stain was removed. However, the sections could be restrained if taken back to 70 per cent alcohol. If the tissues were dehydrated after the pyridine, alcohol or benzol and decalcification treatments, and mounted in wax, the Sudan black staining failed completely.

TOLUIDINE BLUE. Sections were stained with 0.25 per cent toluidine blue in 0.25 per cent borax solution for 15 seconds, and, after they were washed, they were examined mounted in water.

Other Methods. The methylene blue extinction method, as described by Pearse,[13] was employed, using a sodium acetate-sodium barbiturate buffer at pH values from 2.6 to 7.2. The perchloric acid technic for the removal of ribonucleic and desoxyribonucleic acids, Hale's colloidal iron and the Alcian blue method were also used in accordance with Pearse's instructions. For the Alcian blue method, tissues were fixed in Bouin's solution. The periodic acid-Schiff reaction was used on some of the material, and several sections were stained with hematoxylin and eosin. The von Kossa method was applied to the undecalcified epiphyses of rachitic rats. Benditt and French[1] found that N/10 soda removed hexosamine from tissues, and, following their technic, some of the sections were incubated in N/10 soda for 2½ hours at 37° C.

Hyaluronidase. This enzyme was obtained as Hyalase (Benger Laboratories, Ltd.) and was dissolved in 0.1M acetate buffer at pH 5.5, the concentration being 1,500 I.U. of testicular hyaluronidase per ml. Sections were incubated in this solution at 37° C. for 2½ hours, controls being placed in buffer solution only.

Rickets. In order to observe the changes when calcification was interfered with grossly, rats were placed on the Steenbock-Black diet[18] when they weighed from 50 to 60 Gm. After 28 days they were sacrificed or dosed with vitamin D and examined by the methods described above.

RESULTS

Sudan Black Staining. When the bones were stained after decalcification without any preliminary treatment with pyridine, alcohol or benzol, no specific staining of bone or cartilage was seen, all being a uniform pale-blue color. The only areas that stained strongly were where fat was found, as, for example, in the marrow. After treatment with pyridine, alcohol or benzol, quite different results were obtained with both formalin or alcohol fixation.

Intramembranous Bone. In all places where apposition of bone was occurring, a narrow, dark-blue line was found, not on the edge of the bone, but separated from it by a narrow preosseous matrix, the width of this matrix varying from bone to bone, presumably with the speed of apposition. The rest of the bone was quite unstained. This picture was seen most clearly in the rat in the horizontal process of the palatine bone (Fig 1) and also on the endosteal side of the tibial shaft. It was also seen well in the fetal human jaw bone. The writer has called these sudanophil zones "calcification lines."[9]

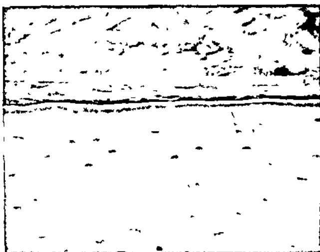


FIG. 1. Horizontal process of palatine bone stained with Sudan black after hot pyridine treatment. Note the sudanophil line (arrow) at the junction of the preosseous matrix and calcified bone. (× 220) (Irving, J. T.: Arch. Oral Biol. 1:96)

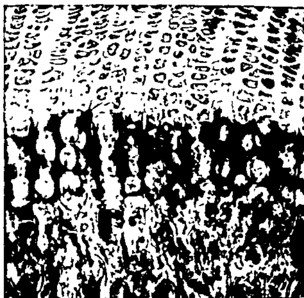


FIG. 2. Upper tibial epiphysial cartilage stained with Sudan black after hot pyridine treatment. Note the staining of the matrix between the last 2 and 3 hypertrophic cells. (× 220)

The calcification lines stained equally well after pyridine, alcohol or benzol treatment. In fact, if the tissue was fixed for a sufficient time in cold absolute alcohol, no further treatment was necessary.

Epiphysial Cartilage. When sections were fixed in formalin and treated with pyridine, alcohol or benzol, the matrix between the hypertrophic cells, just where calcification started, stained a deep blue black, the rest of the cartilage being unstained (Fig. 2). The stain was found only to the depth of 2 or 3 cells, and the cartilage matrix in the diaphysis, on which bone had been deposited and which is so conspicuous in hematoxylin and eosin sections, was not stained. When alcohol-fixed material was stained after treatment with hot alcohol or hot benzol, the appearance was similar but the staining was much less intense, the cartilage matrix being of a greenish color.

It was essential for the above pictures for treatment with pyridine, alcohol or benzol *to precede* decalcification. If decalcification was carried out first, no such staining occured However, decalcification was not essential, as the writer has got identical results with undecalcified rat incisor teeth after pyridine treatment [9] Dehydration was not the cause of the staining An undecalcified section was dried out completely at 60° C. before staining, but no typical Sudan black staining was seen. Nor was heat the cause, since heating in formol-saline for 24 hours at 60° C. was ineffective.

In routine laboratory practice the use of pyridine is to be recommended as being the quickest and the most convenient.

Metachromasia. Since it seemed to be not unlikely that Sudan black was staining some form of acid mucopolysaccharide, toluidine-blue staining was employed. The sections were examined in water, since, as is well known, the color of the tissues is quite changed if alcohol dehydration is carried out. Sylvén[10] has criticized this method on the grounds that the metachromasia seen in aqueous solution is "false." However, Bélanger, in a private communication, has informed the writer that when following the synthesis of sulfomucopolysaccharides in autoradiographs using S^{35} he could not duplicate the results with toluidine blue unless the sections were studied in water or crown oil.

With all methods of preparation and fixation, and whether pyridine was used or not. the calcification lines were strongly meta-

chromatic. But whereas Sudan black staining was restricted to the calcification lines, toluidine blue also stained the preosseous matrix and the rest of the bone. These parts were also metachromatic but less than the calcification lines. This picture is very similar to that described by Vincent[20] in the osteones of mature bone.

The epiphysial cartilage and the cartilage remains in the trabeculae of the spongiosa were very metachromatic in decalcified material not treated with pyridine, but no specific staining was seen corresponding to the area stained with Sudan black. If the bones were fixed in formalin and then treated with pyridine, alcohol or benzol before decalcification, then, while the epiphysial cartilage and the cartilage in the trabeculae were metachromatic, the matrix round the hypertrophic cells was extremely metachromatic and stood out most prominently. Somewhat more of the matrix round the hypertrophic cells was metachromatic than sudanophil, the strong metachromasia extending up 4 or 5 cells. As with intramembranous bone, in which Sudan black stained only the calcification lines while toluidine blue stained both these and much more besides, so in the epiphysial cartilage toluidine blue was much less selective in its staining.

With alcohol fixation, followed by hot alcohol or hot benzol, no selective staining of the matrix round the hypertrophic cells with toluidine blue was found. With these technics, Sudan black staining was much reduced.

P.A.S. Reaction. No specific staining was found at the zone of the calcification lines. The epiphysial cartilage was stained a light-pink color, but, with all technics used, the matrix round the hypertrophic cells stained a deeper pink; the cartilage in the bony trabeculae of the spongiosa did not stain more than the bone surrounding it.

Hale's Colloidal Iron and the Alcian Blue Technics. Neither of these proved to be helpful Hale's method stained bone uniformly blue, and Alcian blue stained only the preosseous matrix Similar results have been reported by Vincent.[20] Both stained the epiphysial cartilage a uniform blue color.

Perchloric Acid Treatment. This did not abolish the Sudan black staining or the metachromasia of the calcification lines and the epiphysial cartilage matrix.

Hyaluronidase. This enzyme had no effect upon the Sudan black staining or the metachromasia of the calcification lines or of the rest of the bone, irrespective of the preliminary treatment. Vincent[20] has also reported that the metachromasia of osteones was unaffected by the enzyme.

The Sudan black staining of the matrix round the hypertrophic cells of the epiphysial cartilage was quite unaffected by the enzyme, irrespective of the preliminary treatment. When the bones were fixed in formalin and treated with pyridine, hyaluronidase removed completely the strong metachromasia seen round the hypertrophic cells, but that in the rest of the cartilage and in the trabeculae of the spongiosa was unaffected. At the same time, the degree of staining with the P.A.S. reaction of the matrix round the hypertrophic cells was increased significantly. The enzyme had no effect on the metachromasia of the matrix of tissues fixed in formalin and treated with hot alcohol or hot benzol, or on that of tissues fixed in alcohol and treated similarly. Thus, under certain conditions, pyridine made the metachromatic material round the hypertrophic cells sensitive to hyaluronidase.

Methylene Blue Extinction Method. This method has been criticized considerably, especially by Gomori,[6] and the present writer has found this technic to produce the most puzzling results. By altering the fixative or the preliminary treatment any picture can be obtained, and it is doubtful if at present the method has any value until the procedures used have been tested rigorously and standardized. Therefore, the results obtained will not be mentioned in any detail.

The sudanophil areas in both bone and epiphysial cartilage could be made to stain at pH 2.6 and, therefore, have a very low



FIG. 3. Upper tibial epiphysis of old rat stained with Sudan black after hot pyridine treatment. The cartilage matrix does not stain, but the cells are filled with sudanophil granules. (× 220) (Irving, J. T.: Nature 183:1734)



FIG. 4. Upper tibial epiphysis of rat whose growth is slowing. Stained with Sudan black after hot pyridine treatment. Both the matrix between the hypertrophic cells and the granules in the cartilage cells are sudanophil. (× 220)

methylene blue extinction value, the rest of the bone and the cartilage being unstained. Unfortunately, different technics had to be used in the 2 cases to get this result. The calcification lines stained at pH 2.6 in tissues fixed in formalin and treated with pyridine, alcohol or benzol, the rest of the bone being unstained. With no preliminary treatment, or with alcohol fixation, the calcification lines did not stain at any pH.

The hypertrophic matrix of the epiphysial cartilage, on the other hand, stained at pH 2.6 after any treatment in which alcohol was involved, the rest of the cartilage being unstained. With either no preliminary treatment or after pyridine treatment, *all* the epiphysial cartilage stained at pH 2.6.

Old Rats. The upper end of the tibia of old rats was also examined. This epiphysis does not close; it is walled off with bone, but the cartilage persists When stained with Sudan black after pyridine treatment, no staining of the matrix round the cartilage cells was seen; however, the cells were filled with granules, which were strongly sudanophil (Fig. 3), but did not stain with toluidine blue or with the P.A.S. reaction. The cartilage was very metachromatic, but this was unaffected by hyaluronidase.

One rat was examined when growth was slowing gradually. The matrix round the hypertrophic cells was still sudanophil, but, in addition, sudanophil granules had begun to accumulate in the cartilage cells (Fig. 4).

Rickets. Young rats were placed on the Steenbock-Black diet for 28 days. Some then were killed and examined, and others were given 30 I.U. of vitamin D by mouth as 1 dose and killed at daily intervals thereafter up to 8 days. In undosed rachitic animals, wide osteoid seams were found in both the tibia and the palatine bones. These



FIG. 5. Horizontal process of the palatine bone of rachitic rat, stained with Sudan black after hot pyridine treatment. Note sudanophil line at junction of osteoid with calcified bone (arrow). Compare with Figure 1. (× 220) (Irving, J .T.: Arch. Oral Biol. 1:96)



FIG 6. Horizontal process of palatine bone of rachitic rat, 6 days after vitamin D dosage, stained with Sudan black after [illegible]

1:96)

were bounded by a sudanophil line where they abutted on calcified bone (Fig. 5). From 2 days after dosage the sudanophilia began to spread from the calcification line through the osteoid as the osteoid calcified



FIG. 7. Upper tibial epiphysis of rachitic rat stained with Sudan black after hot pyridine treatment. Complete absence of sudanophilia of cartilage matrix. (× 220) (Irving, J. T.: Nature 183:1734)

(Fig. 6), but 8 days after dosage calcification cannot have been complete, as the osteoid was still sudanophil.

In fully developed rickets, the sudanophil material disappeared from the epiphysial cartilage matrix (Fig. 7). Many of the hypertrophic cells contained sudanophil granules, which were not seen in normal cartilage of young rats. There was no specifically marked metachromasia of any part of the wide cartilage. Three days after vitamin D dosage, the sudanophil material returned at the time that calcification of the cartilage recommenced, as visualized by the von Kossa technic, and was in the same position in the cartilage. With the progress of time, the von Kossa stained zone widened considerably toward the metaphysis as calcification became more extensive, but the Sudan black staining was restricted to the zone of first calcifying cells and, while wider than in normal calcification, was seldom wider



FIG. 8. Upper tibial epiphysis of rachitic rat 7 days after vitamin D dosage. Stained with Sudan black after hot pyridine treatment. Note reappearance of sudanophil material in matrix round hypertrophic cells. (× 220) (Irving, J. T.: Nature 183:1734)



FIG. 9. Same epiphysis as in Figure 8, stained by the von Kossa technic. Note how much wider the silver-staining zone is than that stained with Sudan black. (× 220)

than 12 cells in thickness and was often less (Figs. 8 & 9). The stain was being taken up in the area where calcification was being initiated, as in the normal process, and it might be expected that in healing rickets this area would be wider.

The parts of the epiphysial cartilage that stained with the von Kossa method were much more metachromatic than the rest of the cartilage, and the metachromatic zone was of much wider distribution than that of Sudan black staining. The metachromasia round the hypertrophic cells was removed by hyaluronidase after pyridine treatment, but that of the cartilage in the trabeculae of the metaphysis was unaffected.

DISCUSSION

In some respects the above results are confusing and contradictory. The chief difficulty in the present investigation was the lack of histochemical methods for specific mucopolysaccharides.

It seems that the following can be concluded safely: the sudanophil substance is connected with the lime salts, since it is removed with them during histologic decalcification unless treatment with pyridine or the other reagents used is carried out first. This somehow renders the substance resistant to decalcification. It is definitely connected with calcification, and almost certainly the very early stages of this, since it is found at the edge of the preosseous matrix, round the hypertrophic cells of the epiphysial cartilage where calcification starts, and is also found in teeth at the dentin-predentin junction and in enamel where it begins to be acid soluble.[9] It also disappears from the epiphysial cartilage during rickets and reappears after vitamin D dosage. It is of

interest that when bone calcification stops, as the epiphysis becomes inactive, the cartilage cells at the head of the tibia accumulate sudanophil granules, and the same happens to the cartilage cells during rickets.

The great problem is what it is. The perchloric acid technic seems to rule out ribonucleic and desoxyribonucleic acids. The substance is clearly not lipid in nature, since it stains only after treatment with fat solvents. The use of pyridine was suggested originally by the work of Hadidian and Pirie,* who found that pyridine precipitated hyaluronic acid. It soon became clear that this was not the reason for the staining, since both hot alcohol and benzol were equally effective in most cases. Nor was dehydration or heat the cause of the staining. The binding of Sudan black to the sudanophil substance must be of a loose nature, as the stain is removed easily by alcohol. At present it is impossible to state what these compounds do to make this particular zone stain.

The next question is whether or not the evidence points to a mucopolysaccharide. While toluidine blue and Sudan black stain similarly, there is a good deal of difference in their staining, toluidine blue always staining far more than Sudan black. It is possible that toluidine blue is less selective and stains several mucopolysaccharides, including one stained only by Sudan black. But if this is so, it is difficult to see why hyaluronidase can remove the metachromatic substance from the epiphysial cartilage, leaving Sudan black staining unchanged. Testicular hyaluronidase is capable of hydrolyzing the chondroitin sulfate component of cartilage [11] Greulich and Friberg[7] showed very clearly with autoradiographs that testicular hyaluronidase removed S^{35} from the epiphysial cartilage when sections were incubated with the enzyme. Although it was not mentioned, presumably the metachromasia of the epiphysial cartilage was removed at the same time. Thus it would appear that Sudan black, if it reacts with the acid mucopolysaccharide molecule, does not do so with the sulfate group, which is the cause of the metachromasia. In all cases where Sudan black did not stain the matrix between the hypertrophic cells, the metachromasia was unaffected by hyaluronidase. After alcohol fixation, when the Sudan black staining of the hypertrophic matrix was reduced, the matrix exhibited no specific metachromasia, and this metachromasia was unaffected by hyaluronidase. Thus it would seem that Sudan black does stain a substance sensitive to hyaluronidase.

It had been hoped that the methylene blue extinction method might be of help. Pearse says that this method, if nucleic acids can be excluded, is specific for sulfate groups and, thus, acid mucopolysaccharides, if the binding of methylene blue occurs at pH values below 4. This undoubtedly occurred under certain conditions. Methylene blue was the only stain that reproduced the same picture as Sudan black, but the preliminary treatment had to differ with bone and cartilage. It seemed that methylene blue was not staining the substance or the same part of the molecule of the substance that stained with Sudan black. The fact that the calcification lines in bones fixed in alcohol, which is an excellent precursor for Sudan black staining, had no methylene blue staining seems to indicate that the chemical or physicochemical changes that make the substance stainable with Sudan black do not apply in the case of methylene blue. Nor could it readily be seen why alcohol treatment *prevented* the bulk of the epiphysial cartilage from being stained by methylene blue at low pH levels. The present writer inclines to the opinion that the methylene blue extinction results support the view that a mucopolysaccharide is involved, but he thinks that, as a whole, the method is as yet unreliable.

The results with the P A.S technic would support the concept that the matrix round the hypertrophic cells contains mucopolysaccharides. The fact that the staining was intensified by hyaluronidase might indicate that the enzyme, as it abolishes the meta-

chromasia, removes groups (possibly sulfates) that block the groups reactive with the P.A.S. reagents. The metachromasia of the calcification lines (and also of the dentin-predentin junction and enamel of teeth) was not affected by hyaluronidase; nor did those areas stain with the P.A.S. method.

The P.A.S. reaction, the methylene blue extinction method and the action of hyaluronidase all indicate that there are differences in the properties of the sudanophil substance in cartilage and bone. The sudanophil substance in teeth behaves exactly as does that in bone. Seeing that the processes involved in endochondral calcification differ in many ways from those in intramembranous bone formation, these differences are perhaps to be expected.

Along with the fact that toluidine blue stains more widely than Sudan black are the observations of Bélanger[3] on the distribution of S^{35} in bone. He found that the zone of S^{35} that appeared under the periosteum after administration of this isotope moved away from the surface of the bone with fresh apposition. Thus, if Sudan black is staining a sulfomucopolysaccharide, some chemical change takes place whereby it is no longer sudanophil shortly after it is laid down. There is no doubt that some change occurs where the preosseous matrix becomes calcified. This matrix has been described by Vincent[20] and Scott and Pease.[14] Vincent says that it is orthochromatic and bounded by a zone of calcified bone (as shown by roentgenograms) that is metachromatic; Lacroix[10] has stated that a "new layer of bone changes its orthochromasia into metachromasia when it begins to manifest a strong affinity for calcium" Some very significant change occurs, presumably in the mucopolysaccharides, at this point. Spicer and Bryant[17] have adduced evidence that the chondroitin of the epiphysial cartilage at the line of endochondral ossification is different from that of other areas, presumably due to its relation with ossification

Solomons and Irving[16] have shown that the collagen of bone and dentin differs from that in soft tissues in that the ϵ amino-groups of lysine and hydroxylysine will not react with fluorodinitrobenzene in fully calcified tissues but are almost all available after decalcification with acid. They postulated that a specific collagen was required for calcification. The present results show that a particular substance, probably a mucopolysaccharide, is associated with the initiation of calcification. Taken in conjunction with the work of Solomons and Irving, the present writer holds the concept that a specific mucopolysaccharide is needed as a primer for calcification and that a specific collagen is required as a seeding medium for the apatite crystal and, later, as a scaffold to which it adheres in fully calcified bone. Possibly, after calcification has started, the mucopolysaccharide is removed as being no longer necessary.

SUMMARY

1. Bone was treated, after fixation, with hot pyridine, hot absolute alcohol or hot benzol, decalcified, mounted in gelatin and sectioned on a freezing microtome.

2. When sections were treated with Sudan black B, only areas where calcification was being initiated were stained, the rest of the hard tissue being colorless. The areas stained were the matrix between the last hypertrophic cells of the epiphysial cartilage and the junction of the preosseous matrix with calcified bone. In rickets the sudanophil material disappeared from the epiphysial cartilage and reappeared soon after vitamin D dosage at the same time that calcification recommenced.

3. Treatment with pyridine or the other reagents had to precede decalcification for Sudan black to stain in this manner. In certain circumstances the sudanophil areas were metachromatic and had a methylene blue extinction value below pH 2.6 with some of the technics used. The sudanophil material

in the epiphysial cartilage differed in some of its properties from that in bone.

4. On the balance of the evidence it is concluded that the sudanophil material is a mucopolysaccharide concerned specifically with the initiation of calcification.

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Alterationes Histochimic in le Stadios Initial del Calcification

Summario in Interlingua

Es describite un methodo pro le tinctura-tion de sitos in osso ubi le processo del cal-cification ha comenciate. Le methodo es etiam applicabile a dentes. Le specimens de tissu esseva fixate in formalina o alcohol absolute e alora tractate con pyridina, alcohol absolute, o benzol a 60 C durante un intervallo de 24 horas. Post le lavage, le tissus esseva discalcificate con acido o acido ethylenediamino-tetraacetic, includite in gel-

atina, e secate con un cryomicrotomo. Postea illos esseva tincturate con nigro Sudan B in 70 pro cento de alcohol e montate in gelea de glycerina. Le matrice circum le ultime cellulas hypertrophic del cartilagine epiphyseal e le junction del matrice preossee con osso calcificate reageva per tincturar se blau obscur, durante que le resto del cartilagine e osso remane incolor. In rachitis le material sudanophile dispareva ab le cartilagine epiphyseal sed re-appareva tosto post le administration de vitamina D, al mesme tempora como le recomenciamento del processo calcificatori.

Un tractamento con pyridina o le altere reagentes debeva preceder le discalcification a fin que le tincturation poteva succeder. Sub certe conditiones le areas de sudanophilitate esseva metachromatic e habeva, sub le conditiones de certes del technicas usate, un valor de extinction de blau methylenic de infra pH 2,6. Esseva notate certe differentias in le proprietates tincturatori de cartilagine epiphyseal e de osso, sed—post ponderar omne le observationes—le conclusion pareva justificate que nigro Sudan B tincturava un mucopolysaccharido que exerce un function in le prime stadios del calcification.

11

Mechanisms of Nuclei Formation in Mineralizing Tissues

ALBERT E. SOBEL, MARTIN BURGER AND SIDNEY NOBEL*

THEORIES OF NUCLEI FORMATION

A fundamental step in the process of mineralization is the formation of nuclei. Nuclei formation may be described as "the process of generating within a metastable mother phase the initial fragments of a new and more stable phase capable of developing spontaneously into gross fragments of the stable phase. Consequently, nucleation is a study of the initial stages of the kinetics of such transformation.

"Nucleation, like ordinary chemical kinetics, involves an activation process leading to the formation of unstable intermediate states known as embryos. The critical rate determining embryo is called a nucleus. A nucleus or germ differs from an equal number of normal molecules in possessing an excess of surface energy sufficient to produce the aggregate as a new phase in the presence of the mother phase."[37] In the case of a solid coming out of solution, such as bone crystal, the metastable phase is a supersaturated solution with respect to the first solid to be formed, and the initial fragments are the solid nuclei of crystal growth.

The subject of nuclei formation was treated recently by Ginell, who developed a generalized theory of association of particles of various degrees of complexity (1-mer, 2-mer, . . . j-mer).[18,19] Of the many possible associations, only certain configurations are capable of acting as a prenucleus of crystal growth. The formation of this critical association of particles requires energy.

While there is a great deal of empirical information on how to produce nuclei,[81] no theory of nucleation of a solid from a solution is available. Not until such a theory of nucleation is developed can the formation of nuclei or templates for the mineralization of hard tissues be understood.

Some of the general conditions required for nuclei formation apply to bone. To accomplish nucleation for bone mineral deposition, one must have a metastable phase, supersaturated with respect to the initial fragments of the solid nuclei. In addition, nucleation in the living organism would depend on controlling the probability that a minute cluster of ions of the right shape exists in the solid state for a sufficient length of time to be the nucleus of crystal growth.

Best[11] makes the point that the chemical potential required for formation of nuclei (microaggregates) is greater than that for crystal growth. Thus, the chemical potential of the solution may be such that growth of the crystal can take place without the formation of new nuclei. For nucleation, the fol-

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lowing relation among chemical potentials of the supernatant and solid must exist:

$$\mu \geq \mu_m > \mu_s > \mu_i$$

where μ is the chemical potential of the supernatant; μ_m *is the chemical potential of* the nucleus (micraggregate); μ_s is the chemical potential of the surface of the crystal; μ_i is the chemical potential of the interior of the crystal.

Although there is evidence of the existence of nuclei in the preliminary stages of calcification,[68,77] there is a gap in available information on the rate of nuclei formation and the minimal dimensions of nuclei of bone crystals. Several equations for the rate of nucleation[8,11,32,81,83] and one equation for the radius of a nucleus[32] are given below to call attention to the type of problem to be encountered in studies of equilibria, kinetics and geometry of nuclei formation in bone and teeth.

A. Equations for the Formation of Nuclei

Equation I, which follows, was established by Becker and Doering for nucleation of a liquid from the vapor phase.[8] It is given as modified by Pound.[49] This equation is accepted by almost all authorities and serves as a point of departure for further speculation.

$$\text{I.} \quad J = \frac{\alpha\sqrt{2} \times N^{3/2}}{\sqrt{\pi} \times R^2}\left(\frac{P_\infty}{T}\right)^2 \frac{\sqrt{\sigma}M}{\rho}\left(\frac{P_1}{P_\infty}\right)^2 \times \exp\left[\frac{-16\pi M^2\sigma^3}{3\rho^2 k^1 T\left(RT\ln\frac{P_1}{P_\infty}\right)^2}\right]$$

The exponential term is the free energy of activation of the process.

P_1/P_∞ is the supersaturation ratio.

J = rate of nucleation, expressed as *nuclei/cc./second.*

P_1 = partial pressure of vapor.

P_∞ = equilibrium vapor pressure of liquid.

α = condensation coefficient.

σ = surface tension of liquid.

ρ = density of liquid.

$k^1 = R/N$.

M = molecular weight.

R = gas constant.

N = Avogadro's number

A modified form of the foregoing equation, for nucleation of a solid from the liquid phase, applied more particularly to barium sulfate,[32] is given in the following equation:

$$\text{II.} \quad I \approx K_v \exp\left[-a\sigma^3 v^2/k^3T^3(\ln S)^2\right]$$

where

I = rate of nucleation.

$K_v = n\nu \exp[-\Delta G_A/kT]$

a is a geometric factor.

σ = interfacial energy per area between nucleus and solution.

v = volume per "molecule" of $BaSO_4$ crystal.

S = critical supersatu[illegible] $(K_{IP}/K_{SP})^{1/2}$, where K_{IP} is the [illegible] and K_{SP} is the solubility pro[illegible]

n = number of Ba^{++} and SO_4^{--} ions per volume.

ν = jump frequency.

ΔG_A = free energy of activation for growth of crystals.

"This equation is difficult to apply because the energy terms are not well defined. For example, there are little available data on the surface energy of solids, further, the surface [illegible] [illegible]ries from plane to plane. The structu[illegible] small nucleus is not yet *known*[illegible] *is a polyhedron,* the contribution [illegible] and corners to the interfacial [illegible]. The [illegible], is also [illegible] because cr[illegible] invo[illegible]n and a

face processes, each of which has a characteristic activation energy."

The conditions for the spontaneous formation of nuclei from a supersaturated solution are defined in Equation III, quoted from Weissberger.[11]

III. $$N = ce^{-Q/kT}e^{-w/kT}$$

where

N = rate of nuclei formation.

c = constant.

Q = activation energy for diffusion.

K = Boltzman constant.

T = absolute temperature.

w = work required to form the surface of the nucleus.

It follows from this equation that, given sufficient time, nuclei formation will occur even with a low degree of supersaturation. To accomplish nucleation in a reasonable time, either a very high degree of supersaturation or a catalyst of nuclei formation is usually needed. Such a catalyst can be a solid surface that has some aspects of the crystal lattice and, in effect, is the equivalent of a seed. This type of seeding is referred to as epitaxis. The rate of nucleation, I_{κ}, by epitaxis is discussed[13] in terms of the following equation.

IV. $$I_{\kappa} = f(\alpha\delta\beta)$$

where α = degree of supersaturation.

δ = disregistry between the crystal lattice of the nuclei-seeding solid and the forming crystal. This is defined quantitatively as a/a_{α} where a_{α} is the atomic separation in the forming crystal (A), and a is the difference between corresponding atomic separations in A and S, the substrate (epitactic seeding crystal)

β = factor determined by the relative chemistry, bond type, etc., between A and S.

In general, epitactic nucleation involves a structural relationship between the substrate crystal and the new crystal formed. Whether or not such a relationship exists between the organic portion of the calcifying matrix and the structure of the bone crystal formed remains to be established.

Best[11] has presented suggestions concerning the kinetics of formation of calcium phosphate microaggregates in the absence of seeding sites. He visualizes that the reaction is a bimolecular one between a positively charged ion containing calcium and a negatively charged ion containing phosphate. One ionic species is denoted as "i," the other as "j." On this basis he derived the equation given below for the rate of reaction between these ionic species.

V. $$v_{ij} = k_1 e^{-Q_iQ_j/r_aDkT}C_iC_j - \underline{k_1}e^{-Q_iQ_j(1/r_a-1/r_o)DkT}C_{ij}$$

where

v_{ij} = the rate of reaction between the i^{th} and j^{th} species.

$k_1 = \left(\frac{kT}{h}\right)e^{-\Delta F_1^{\ddagger}/kT}$ where the $\Delta F^{\ddagger}$ term involves the free energy of formation of the activated complex from the reactant ions

$\underline{k_1} = \left(\frac{kT}{h}\right)e^{-\Delta F_1^{\ddagger}/kT}$ where the $\Delta F^{\ddagger}$ term involves the free energy of formation of the activated complex from the product.

Q_i = net charge of the i^{th} species.

Q_j = net charge of the j^{th} species.

r_a = mean distance of separation of the charges on the 2 ionic reactants when the activated complex is formed.

r_o = mean distance of separation of these charges in the product of the reaction.

D = effective microscopic dielectric constant of water.

k = Boltzman constant.

C_i = concentration of i^{th} species.

C_j = concentration of j^{th} species.

C_{ij} = concentration of product of union of i^{th} and j^{th} species.

h = Planck's constant.
T = absolute temperature.

The equation resolves itself to a measurement of the difference between the rates of association and dissociation of the reactant species.

B. Equation for the Radius of a Nucleus[32]

VI. $iRT/M \ln S_C = 2\sigma/\rho r$

where

S = supersaturation ratio, defined as above (Equation II).
S_C = critical supersaturation.
i = van't Hoff factor.
M = molecular weight.
σ = interfacial tension.
ρ = density.
r = radius of nucleus.

Experimentally, one determines the time required for spontaneous formation of crystals at various levels of supersaturation and extrapolates to zero time to determine S_C. In employing this equation, one must utilize systems in which impurities that would catalyze the formation of crystals are eliminated. For $BaSO_4$, the radius calculated for the nuclei was 0.01 μ. By applying the above equation it should be possible to determine the diameter of nuclei of bone crystal growth.

THEORETIC CONSIDERATION OF NUCLEI FORMATION IN MINERALIZING TISSUES

Several general mechanisms of nuclei formation may be proposed for bone crystal. One is to increase the degree of supersaturation to the point of spontaneous precipitation. This is the concept on which many of the earlier theories of calcification were based[26,52] Alternately, one may propose that nuclei formation is aided by a catalyst or catalytic system* below the level of spontaneous precipitation in a metastable solution. The catalyst may be either homogeneous, i.e., the catalyst is in solution, or heterogeneous, i.e., the catalyst is a solid. A homogeneous catalyst would probably act by binding specific ions for forming templates of crystal growth or by forming a polymer with such ions followed by decomposition, releasing a nucleus.[82] (The lack of experimental evidence does not preclude the existence of such a system for calcification.) A nuclei-inducing solid may act by binding specific ions for nuclei formation or by providing an epitactic surface, inducing parallel growth of crystals. The latter would take place because the initiating surface has some aspects of the crystal to be formed. (See Equation IV.)

Supersaturation, for an ionic, slightly soluble solid, may be defined as the ratio of activity product to solubility product. However, the solubility product principle, which helps in understanding the formation of slightly soluble ionic salts from 2 ions, appears to be inadequate in defining the solubility properties of a complex solid such as hydroxyapatite, the lattice unit of which is made up of 18 ions. Anomalies of solubility product and the solubility of hydroxyapatite are amply discussed by Neuman.[45] A satisfactory explanation must await new developments in theoretic chemistry.

A fundamental question is whether the first solid aggregate formed in the mineralization of bones and teeth is hydroxyapatite or a simpler calcium phosphate. From kinetic reasoning it is difficult to visualize the direct formation of a solid unit containing the 18 ions required for the hydroxyapatite lattice, $Ca_{10}(PO_4)_6(OH)_2$. In contrast, the formation of $CaHPO_4$, or some other simple calcium phosphate, would require collision of only 2 ions per unit. Shear and Kramer proposed that $CaHPO_4$ was the first solid formed in calcification.[56] McLean's studies at physiologic pH levels indicate that $CaHPO_4$ is indeed likely to be the first aggregate in calcification in vitro.[43] Studies by

* A catalyst or catalytic system is defined as any substance or system that alters the velocity of nucleation

our group support McLean's findings.[74] Best[11] expanded on this concept and suggested that a simple calcium phosphate moiety produced by a bimolecular reaction might be the first aggregate, pointing out that (a) the probability of 3 or more individual ions undergoing simultaneous collision could be neglected, and, therefore, an aggregate comprised of several calcium and phosphate ions must be built up by a process involving the collision of only 2 ions; (b) the probability of a step involving 2 ions of like charge could be neglected; (c) the probability of a step involving the union of 2 ions of unlike charge would be relatively large.

Another fundamental question, What is the minimal chemical potential required for nuclei formation?, has not been answered experimentally. The answer would depend on determining thermodynamic equilibrium between the nuclei and the liquid phase. We know that $\mu_{soln} \geq \mu_{nuclei} > \mu_{crystal\ surface} > \mu_{crystal\ interior}$, where μ is the chemical potential or free energy.[11] In the solution, μ is a function of $[A_{Ca^{++}}]\ [A_{HPO_4^{=}}]$, generally referred to in its simplified form as Ca $\times$ P product. This minimal Ca $\times$ P product might be calculated if we knew the free energy of the nucleus compared with the free energy of the crystal surface and if the solubility product of the bone crystal at a given size could be established. The increase in supersaturation required for nuclei formation would be a function of the difference between the respective free energies of the nuclei and the crystal surface. (Another factor here would be the energy of formation of the nucleus.) In the case of bone crystals, the energy difference may be a large one. Thus, the Ca $\times$ P required for nuclei formation may be very large compared with that for crystal growth. There is a general error in considering the solubility of hydroxyapatite in establishing the conditions required for nuclei formation in bone. Neuman has proposed that, since the body fluids are supersaturated with respect to hydroxyapatite, the task of the calcifying mechanism is merely the formation of nuclei.[60,65] This conclusion cannot be drawn until the minimal degree of supersaturation required for the formation of nuclei is known. If this critical product of calcium and phosphate ions is higher than that which exists in the body fluids, then the task of the calcifying mechanism would be the concentration of calcium and/or phosphate prior to the formation of nuclei.

The accumulated empirical evidence that led to proposing $CaHPO_4$ as the first aggregate in calcification[13,56] is now interpreted to mean that the solubility product of $CaHPO_4$ may approximate the degree of metastability required for the formation of nuclei. The solubility product of $CaHPO_4$ is considerably higher than the Ca $\times$ P of the body fluids in general. It must be borne in mind that if this interpretation is correct, then the local concentration of calcium and phosphate above this level of supersaturation need last only long enough to produce nuclei, since crystal growth of the new solid phase (ultimately hydroxyapatite) proceeds at a lower Ca $\times$ P product.

Macromolecules such as collagen are known to induce the formation of nuclei for the growth of hydroxyapatite crystals.[3,5,6,20,35,63,80] While this may not be the complete calcifying system, understanding the nature of this phenomenon as related to the properties of macromolecules appears to be a critical step in understanding the similar process in vivo. The characteristics of macromolecules, particularly polyelectrolytes, permit consideration of mechanisms other than epitaxis These large molecules can have sites of high-charge density arising because the ionized groups are forced by their covalent binding to remain in close proximity to each other.[79] Oppositely charged ions would have a relatively high concentration around the area of high-charge density, decreasing gradually with distance. Other internal properties of polyelectrolytes also may be considered. Supersaturation could be

enhanced by the rearrangement or depolymerization of macromolecules that bind ions. For example, the binding of cupric ion to polyacrylic acid is approximately 10^4 times that of the binding of copper to the low molecular weight analogue glutaric acid.[21] Polyacrylic resin polymer has an association constant with calcium of 100, while the association constant of calcium with the monomer acetic acid is negligible.[25] Thus, an appropriate rearrangement or depolymerization could lead to the alternate binding and release of ions. More intensive studies with simple polyelectrolyte models such as polystyrene sulfonic acid and polyamino acids, when combined with concepts of the mechanism of nuclei formation, and possibly nuclei growth, may provide a fundamental clue to the process of calcification.

INVESTIGATION OF THE NUCLEATING SYSTEM IN MINERALIZING TISSUES

In Vitro Calcification; a Tool for Studying Nuclei Formation

A great many studies, undertaken to elucidate the mechanism of calcification, have been carried out by placing sections of rachitic bone cartilage in solutions resembling the extracellular fluids or blood serum. Histologically, the resulting mineralization resembles healing of rickets in vivo.[52,57] X-ray diffraction studies and analyses of the chemical composition of sections calcified in vitro also show marked resemblance to in vivo calcification.[28,30] The mineralization takes place selectively in the matrix of the hypertrophic epiphysial cartilage and, when the Ca $\times$ P product is high enough, in the region referred to as bare osteoid. Characteristically, no mineralization is found in the nonossifying proliferating preosseous cartilage. The readiness with which new mineralization was observed under a wide variety of conditions encouraged the use of this type of experiment to elucidate the intimate mechanism of mineralization. The main objection to this type of experiment is that hypertrophic cartilage matrix is not identical with the cartilage of normal bone. Moreover, the objection has been raised that the mineralizing mechanism of bone, enamel and dentin is not identical with the system responsible for the mineralization of preosseous cartilage. Nevertheless, if one understood completely the mechanism of calcification of hypertrophic cartilage in the in vitro system, it might be possible to utilize this information to understand the calcifying mechanism elsewhere. As will be seen below, several characteristics of the mechanism of calcification were elucidated by such experiments.

Nucleation in Preosseous Cartilage

When a rachitic bone section is exposed to calcifying solution for a short time, no change is visible under microscopic examination following silver staining However, a drastic change has taken place; namely, the formation of submicroscopic solid aggregates for the deposition of mineral salts,[47,68,77] representing the early stages of nuclei growth. Robinson's recent paper indicates that these submicroaggregates may be visible under the electron microscope.[50] Such sections, to which we refer as "nucleated," mineralize in vitro after one of the following treatments, while nonnucleated sections, under the same conditions, show lack of mineralization (Table 1): (a) heating in distilled water at 65 to 77° C. for 10 minutes; (b) shaking in distilled water at room temperature for a half hour, (c) incubation in a calcifying solution containing magnesium and iodoacetate ions; (d) x-ray irradiation with 1,000,000 R;[29] (e) incubation in strontifying solution.[48,75] "Nucleated" sections mineralize at a lower Ca $\times$ P product than would be necessary to initiate calcification in a fresh section.[47,68,77] (Table 1). With the above tests it was possible to demonstrate that nucleation could occur in vivo in rachitic animals after as little as 10 to 20 hours of starvation.[59,77] Moreover, by means

TABLE 1. TESTS FOR "NUCLEATED"* EPIPHYSIAL HYPERTROPHIC CARTILAGE[29,49,68,77]

	Degree of Calcification† After 18 Hrs. in Calcifying Solution	
	"Nucleated" Sections‡	Sections Not "Nucleated"
Heated 10 min. at 76-77° C. in distilled water ...	2(++++)	0(0)
Shaken 1 hr. in distilled water at room temperature	1.5(++++)	0(0)
1 mM. Mg^{++} and 3 mM. IAc^{-} in calcifying solution	1.5(++++)	0(0)
Strontifying solution where Sr = 20, P = 20	2(++++)	0(0)
X-ray irradiation 1,000,000 R ..	2(++++)	0(0)
Calcifying solution where Ca × P = 10	Faint trace	0(0)
Calcifying solution where Ca × P = 20	1(++)	0(0)
Calcifying solution where Ca × P = 30	1.2(+++)	Faint trace
Calcifying solution where Ca × P = 50	2(++++)	1.7(++++)

* Sections were pretreated in calcifying solution, Ca = 10 mg.%, P = 5 mg %, for 2 hours @ 37°C., pH 7.3 ± 0.05.

In a rigorous sense, a nucleus of crystal growth is the first solid aggregate that can support crystal growth. It is most likely that in these experiments the procedure for "nucleating" rachitic sections resulted not only in nuclei but also in nuclei at various stages of growth despite lack of visibility with the silver stain

† The degree of calcification is indicated as follows. 0(0) no calcification, 1(+) trace, 1(++) broken thin line, 1(+++) almost complete thin line across provisional zone, 1(++++) thin line across provisional zone, 2(++++) heavy line across provisional zone, including primary tongues of cartilage, 3(++++) heavy line across provisional zone, including primary and seco...

...nucleated sections at 55° C.[47]

of these tests, it was found that the rate of nucleation increases up to a maximum at approximately 55° C., after which there is inactivation at 60 to 62° C., which coincides with the shrinkage temperature of decalcified bone matrix.[47,50]

These experiments establish that the process of crystal growth is separate from nuclei formation and is independent of the nucleating mechanism.

MAGNESIUM-LINKED INHIBITION OF NUCLEI FORMATION

The inhibition of calcification in vitro by strontium, cyanide, iodoacetate or fluoride ions is dependent on the presence of magnesium ions in the solution[21,23,69,71] (Table 2). The inhibition attacks the nucleating mechanism, since, if nuclei are already present (as in sections pretreated with calcifying solution[68]), magnesium-iodoacetate does not inhibit further calcification. Although this has not been established for the other inhibiting ion-pairs, it is probable that they act

TABLE 2. INFLUENCE OF SR^{++}, MG^{++}, F^{-} AND CN^{-} IONS ON CALCIFICATION IN VITRO[21,23,71]

Sr^{++} mM./L.	Mg^{++} mM./L.	Degree of Calcification (Mean) Ca × P = 40	Ca × P = 70
0.58	0.75	0(0)	0(0)
0.58	0	1.1(++++)	
0	0.75	1.1(++++)	
1.33	0		2.3(++++)
0	1.33		1.5(++++)
0	0	2(++++)	2.7(++++)

F^{-} mM./L.	CN^{-} mM./L.	Mg^{++} mM./L.	Degree of Calcification (Mean)
0	0	0	2(++++)
0	0	1.0	1.5(++++)
1.0	0	0	3(++++)
1.0	0	1.0	0(0)
0	1.0	0	2(++++)
0	1.0	1.0	Trace

in a similar fashion. These findings suggest two possibilities: (a) there may be two critical sites in the active centers of the nuclei-forming catalyst; (b) a cluster of the inhibiting pair of ions may be bound competitively, blocking the capture of a calcium phosphate cluster by the nuclei-forming catalyst.

Increase of Ca × P Product in Healing Rickets

The development of the supersaturation theory of nuclei formation in calcification was given impetus by studies designed to elucidate the role of vitamin D in healing rickets. The healing of rickets, which is evidence of nucleation, is favored by an increase in the Ca × P product in the body fluids. Kramer and Howland, in their classic studies, established that the Ca × P product of blood serum was low in rickets and was elevated when healing took place.[37] It was shown by Kramer, Shear and Siegel that, regardless of the method of raising the serum Ca × P product, the healing of rickets followed. The methods that they employed were: administering vitamin D; inducing loss of weight; and changing the Ca:PO_4 ratio of the diet.[34,35]

Further studies show that in vitro calcification depends on a minimal product of Ca × P, which varies considerably with the type of rachitic bone section or in preosseous embryonic bone.[46,52,57,69,70,72,73] The minimal Ca × P product for new mineralization in vitro of embryonic bone is 16;[46] for rachitic bone it is 35;[52,57,58] for beryllium rachitic bone it is 60;[73] and for strontium rachitic bone it is 90.[69,70,72] The minimal Ca × P product of 90 for the healing of strontium rickets in vitro explains why in vivo strontium rickets is not affected by vitamin D treatment. The equivalent serum Ca × P product would be 150, since in vivo some of the Ca is bound to serum proteins Such a product is of too high a magnitude to be reached in rat serum.

Additional proof of the importance of the Ca × P product is the transplantation of rachitic sections under the skins of normal and rachitic rats.[13] Healing takes place in the normal animal or in the rachitic animal treated with vitamin D, but not in the untreated rachitic animal. Healing does not take place in the unmodified blood serum of the rachitic animal, but, after the addition of phosphate to such serum, healing proceeds, although the Ca × P product is still below that found in normal rats of the same age (Table 3).

Thus, the general conclusion can be reached that any factors causing *a rise in the activity product of Ca × P*, when everything else is equal, will enhance nuclei formation, and that increasing the degree of supersaturation *is prerequisite to the formation of nuclei in rachitic cartilage*. Moreover, *"local factors"* in the calcifying matrix can influence the minimal degree of supersaturation required for nuclei formation. One may add that the above evidence indicates that *the primary role of vitamin D in healing rickets is the raising of the serum Ca × P product*, although additional functions of this vitamin are not excluded.

Extension of the Supersaturation Concept

The further development of the supersaturation concept of the mechanism of mineral deposition in bones was based on the assumption that only when a state of supersaturation is created that is higher than exists in the body fluids can bone salt formation begin. The first step visualized is the release of additional inorganic phosphate, which then increases the Ca × P product, causing formation of a nucleus. The Robison scheme[52,53] proposed that the enzyme phosphatase, present in the calcifying matrix, caused the splitting of organic phosphate, converting it to inorganic phosphate, with a resultant rise in the Ca × P product.

Gutman and others have extended Robison's concept by indicating that the glycolytic

TABLE 3. CALCIFICATION OF HYPERTROPHIC CARTILAGE OF RACHITIC TIBIAL SLICES AFTER SUBCUTANEOUS TRANSPLANT TO RATS ON RACHITOGENIC AND NORMAL DIETS, AND AFTER INCUBATION IN INORGANIC MEDIUM AND RACHITIC RAT SERUM[13]

Treatment	Ca, mg. %	P, mg. %	Ca × P	Time	Degree of Calcification
Transplanted subcutaneously to rats on rachitogenic diet	9.8*	1.7*	16.7*	1 wk.	0(0)
				2 wks.	0(0)
				3 wks.	0(0)
Transplanted subcutaneously to rats on normal diet.....	10.3*	8.8*	89.8*	1 wk.	1.3(+++)†
				2 wks.	2.5(++++)†
				3 wks.	3.5(++++)†
Inorganic medium	4.0	5.0	20.0	18 hrs.	0(0)
	10.0	5.0	50.0	18 hrs.	1.5(++++)
Rachitic rat serum	10.6	1.5	15.9	18 hrs.	0(0)
Rachitic rat serum + PO_4...	10.6	5.0‡	53.0	18 hrs.	1.1(++++)

* Ca and P were determined in blood serum of rats receiving transplants.
† Calcification was observed in the metaphysial osteoid and was most extensive after 3 weeks.
‡ The phosphorus in this serum was raised from 1.5 mg. % to 5.0 mg. % by the addition of inorganic phosphate.

enzymes in preosseous tissue are probably responsible for concentrating phosphate.[26,27,41] By means of inhibitors they provided evidence that the glycolytic cycle was involved in calcification. They proposed that the organic phosphate required for calcification came from glycogen's combining with inorganic phosphate and then entering the glycolytic cycle. The derived phosphate esters provide substrate as well as energy, probably through the splitting of phosphopyruvic acid and the formation of ATP. It is easy to visualize the possibility that the inorganic phosphate ions released by the cycle become more concentrated at the site of calcification than the initial inorganic phosphate that entered the system.

Cartier proposed that ATP was a critical substance in the calcifying mechanism.[15,42] He showed that embryonic bone cartilage mineralized more effectively in the presence of ATP than other organic or inorganic phosphates. Indirect support was lent to this view by evidence of the presence of an abundance of ATP in bone cartilage.[1] Recently, UTP was shown to be even more effective than ATP in producing mineralization.[16] It was implied in some of our observations that ATP might effect a transfer of energy to a system required for nucleation in preosseous tissue.[61] Preliminary treatment with ATP in the presence of calcium ions permitted the subsequent mineralization of the bare osteoid in rickets with a Ca × P product of 10. Without such treatment, the bare osteoid requires a Ca × P product of 70, while mineralization of preosseous cartilage requires a minimum product of about 35. While energy transfer is an attractive explanation, at this writing we cannot eliminate the alternate possibility that the effect is due to direct interaction of the calcium with phosphate liberated by ATP'ase.

OBJECTIONS TO NUCLEI FORMATION VIA ORGANIC PHOSPHATES

Several objections to the above schemes may be raised. A major defect is that it is difficult to visualize a nonspecific system as being responsible for a highly specific process in the body, such as calcification. Phosphatase, the glycolytic cycle and ATP exist in other parts of the body where no mineralization takes place; thus there is failure to

account for the specificity of mineral formation in normally mineralizing tissues. In rachitic cartilage, at least, it has been possible to inactivate calcifiability in the presence of these enzymes and to restore calcifiability in their absence.[14,66,69] Thus it is possible to separate the production of supersaturation via phosphatase and the glycolytic cycle from the minimal system required for calcification. Also, one would expect a system for handling, or possibly even concentrating, calcium ions at the site of calcification.

The Search for a Specific Catalyst of Nuclei Formation

The existence of a specific system that combines with calcium as part of the process of calcification was indicated in our early experiments with strontium rickets. Evidence was presented for the competitive retardation by strontium ions of a system responsible for mineralization.[70] The scheme visualized is given in Figure 1. Using this scheme, it was possible to achieve reversible inactivation of the calcifying mechanism of hypertrophic cartilage in a reversible manner by a variety of other cations.[59,63,74,76] It was also possible to separate calcifiability from the action of the glycolytic enzymes and phosphatase.[14,66,69] Both calcium and strontium ions prolonged the survival of the calcifying mechanism.[22] This stabilizing action is reminiscent of the known protective effect of substrates and some inhibitors on enzymes.

Concepts of Collagen-Mucopolysaccharide Complex in Nuclei Formation

When toluidine blue was employed as an inactivator of the calcifying mechanism, it was observed that metachromatic staining of hypertrophic cartilage increased when 15 mEq./L. of calcium ions was present in the dye solution. By contrast, when polyelectrolytes were used, more particularly chondroitin sulfate, which is present in the ossifying cartilage, the metachromatic color produced with toluidine blue is destroyed by calcium ions. A search was made for a metachromatically active substance that would exhibit the same staining phenomenon observed in rachitic

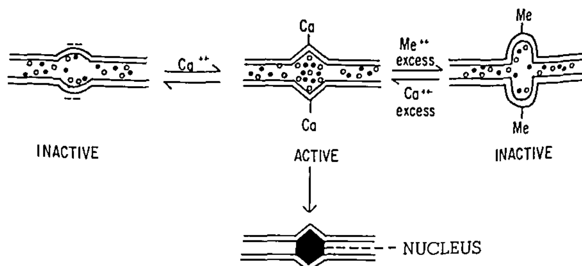


Fig 1 Model of nuclei-forming mechanism including reversible inactivation by calcium-substituting ions The nuclei are formed between the collagen protofibrils at selective sites activated by calcium The complete system is activated by a mucoprotein or mucopolysaccharide and utilizes energy such as is obtained from ATP, UTP, gylcolytic and/or citric acid cycles. The actual mechanism must be visualized as a 3-dimensional process. (Sobel, Laurence & Burger: Tr. New York Acad Sc. 22:237)



FIG. 2. Demineralized rachitic tibia after in vitro calcification at pH 7.5. Note lack of mineralization after silver staining.



FIG. 3. Demineralized rachitic tibia treated with chondroitin sulfate and $CaCl_2$ followed by calcification in vitro at pH 7.5 and subsequent silver staining. Note remineralization of hypertrophic cartilage, epiphysis and diaphysis. Remineralization of epiphysis and diaphysis takes place with chondroitin sulfate alone; hypertrophic cartilage required both $CaCl_2$ and chondroitin sulfate treatments.

bone cartilage. It was found that collagen, interacted with chondroitin sulfate at pH 4, exhibited increased metachromasia in the presence of calcium ions. When such collagen was placed in solutions used for calcification in vitro it mineralized. Therefore, it was proposed that collagen and chondroitin sulfate form a complex homologous to the system permitting the calcification of rachitic cartilage.[58,63]

In an extension of these investigations, attention was turned to the behavior of rachitic bones that had been completely demineralized by means of EDTA. When, subsequently, such demineralized bones were treated with chondroitin sulfate followed by $CaCl_2$, the hypertrophic cartilage and, to some degree, the epiphysial trabeculae and diaphysis, but not the nonossifying cartilage, were found to have remineralized in a histologically valid manner so far as could be determined by the silver nitrate staining method. With only the chondroitin sulfate treatment, the epiphysial trabeculae and diaphysis remineralized to a lesser degree, and the hypertrophic cartilage did not mineralize. Controls that were merely demineralized without additional treatment failed to mineralize (Figs. 2 & 3). The mineralization required a pH of 7.5, whereas calcification of fresh rachitic cartilage takes place at a pH of 7.3. Thus, there appears to be some difference between artificially reconstituted matrix and the actual bone. However, when such chondroitin-sulfate-treated demineralized bone was placed under the skin of animals, it mineralized almost as well as did fresh untreated control tissue sections[12] (Table 4). (Recent experiments indicate that when demineralized bone is treated with chondroitin sulfate and placed in trephined

TABLE 4. CALCIFICATION OF CHONDROITIN-SULFATE-TREATED DEMINERALIZED RACHITIC BONE TRANSPLANTED BENEATH THE SKIN OF NORMAL RATS[61,67]

Treatment of Demineralized Bone	Degree of Calcification (Mean)			
	7 Days	14 Days	19 Days	26 Days
No treatment	0(0)	0.5(++)		3.0(++)
$CaCl_2$	Trace	1.5(+)	3.0(++)	3.0(++)
Chondroitin SO_4	Trace	2.0(+++)		3.0(++)
Chondroitin SO_4 + $CaCl_2$	Trace	3.0(++++)	4.0(++++)	4.0(++++)
Fresh rachitic sections ...	1.3(+++)	2.7(++++)*	4.0(++++)*	

* Were extremely hard; could not be sliced with a scalpel.



FIG. 4. Demineralized jaw after treatment in basal salt solution at pH 8 2, followed by in vitro calcification at pH 7.3 and subsequent silver staining. Note absence of in vitro calcification.

skulls, it causes healing of the defect more rapidly than does either fresh bone or demineralized untreated bone, as shown in Figure 6.[61,62] These observations provoke speculative thought as to whether or not mucopolysaccharides such as chondroitin sulfate represent an osteogenetic organizer in normal bone formation.)

An alternate method of restoring mineralization is to treat demineralized bone or jaw in a basal salt solution at pH 7.6 to 8.2 and follow this by $CaCl_2$ treatment. The bone, hypertrophic epiphysial cartilage and dentin remineralized (Figs. 4 & 5),[60] as measured by silver stain and increase of ash. The enamel and the nonossifying proliferating cartilage did not mineralize.

The above experiments suggest the existence of a specific acid-insoluble protein, probably collagen, as being responsible for nuclei formation. Restoration of calcifiability after demineralization does not take place in the nonossifying cartilage, which also contains collagen, and does, in fact, bind calcium without inducing nuclei formation.[54]



Fig. 6. Roentgenogram of trephined skull of a rat 6 weeks after operation, showing extent of healing of defects following insertion of pieces of demineralized bone tissue. Right side (roentgenogram opaque) contains chondroitin-sulfate-treated EDTA demineralized bone. Left side contains untreated EDTA demineralized bone. (Sobel, A E.: Bull. Jewish Hosp. 1:13)

Mineralization of Collagen

Further investigation has revealed that reconstituted fibers of acid-soluble collagen do not require chondroitin sulfate for their mineralization. Fibers precipitated from acid solution by means of heparin, lauryl sulfate, NaCl or dialysis against Na-acetate solution mineralized about as well as fibers reconstituted with chondroitin sulfate. Thus the conclusion was reached that reconstituted collagen fibers have the capacity to mineralize as an intrinsic property.[3,6,61] Acid-soluble collagen obtained from rat-tail tendon and from rat skin, when purified, mineralized equally well, although in crude preparations mineralization of tail tendon collagen was strikingly superior. These results raised two questions: What is the mechanism of mineralization of collagen? How is collagen mineralization involved in the calcification of bone?

Some clues to the manner in which collagen may function in promoting nuclei formation were observed in the electron microscope studies shown in Figure 7. Acid-soluble collagen precipitated with chondroitin sulfate under the conditions of these experiments is nonstriated. This collagen is transformed to the 640 A spaced striated form by basal salt solution containing either calcium or phosphate. The control, kept in basal salt solution, does not do this.[4,62] From the results obtained in the transformation studies, one may suggest cautiously that either calcium or phosphate ions interact with a critical site in the nonstriated collagen, where an electrostatic bond between positively and negatively charged groups is

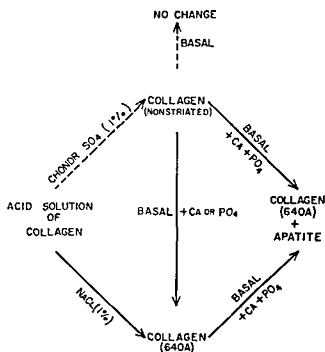


FIG. 7. Influence of calcium and phosphate on the transformation of reconstituted acid-soluble collagen fibers (nonstriated) to the 640 A spaced striated form.[4] (Bachra & Sobel: Proc. Soc. Exper. Biol. & Med. 102:314)

split, allowing the rearrangement of the patterns of aggregation of tropocollagen units to the 640 A spaced fibers. Alternately, but less likely, 2 independent sites, one binding *calcium and the other binding phosphate,* might produce such rearrangement. To illustrate, electrostatic bonds within the collagen fibril that might be interrupted could be between terminal amino and carboxylic groups on the tropocollagen units or, more likely, *side chain bonds between the ξ-amino* groups of lysine or hydroxylysine and δ-carboxylic group of glutamic acid or the γ-carboxylic group of aspartic acid.[7,78] The above calcium- and phosphate-capturing groups in the right configuration may be the *same as those involved in the capture of* prenuclei clusters (see mechanism of nuclei formation below).

A comparison between the mineralization of collagen and in vitro calcification reveals that calcification in vitro takes place at a lower product of Ca × P and proceeds more rapidly.[6,28,61] Even at a Ca × P product 45, calcification in vitro can reach 40 per ce ash in 18 hours, while the calcification collagen tends to be less than 0.1 per ce in the first 24 hours, although it can rea as much as 60 per cent in the second hours.[6] While it is possible that furth purification of collagen may produce a su stance that mineralizes as well as rachit cartilage in vitro, it is unlikely.[3] It is t authors' view that the complete system for vitro calcification is not limited to reconsi tuted acid-soluble collagen fibers but that depends on factors such as (*a*) a speci acid-insoluble collagen, (*b*) a sulfated muc polysaccharide or mucoprotein, (*c*) enzyn systems such as the glycolytic cycle[2] or t *citric acid cycle,*[17] *as well as (d) ener* sources such as ATP[1,15] and/or UTP.[16] W must also consider a system that acts n only as a source of energy for nuclei form tion and additional phosphate but also hitherto undiscovered system for increasi the concentration of *calcium ions.*

THE NUCLEI-FORMING MACROMOLECULE

From these studies, as well as those c Strates *et al.*[80] and Glimcher *et al.*,[20] it ap pears that mucopolysaccharides are not ne essary for the calcification of reconstitute collagen. However, from present evidenc we believe that in preosseous cartilag mucopolysaccharides probably are involve in bringing about the proper structure c acid-insoluble collagen that is responsib for initiating mineralization. The *calcifyin* mechanism, at least in the preosseous carti lage, requires a cluster of negative charge on some protein molecule, probably colla gen. This negatively charged protein, unlik reconstituted collagen, is inactive unt treated with calcium ions (see *Fig. 1*). I the test tube, reactivation of the calcifyin portion of preosseous cartilage demineralize with EDTA at pH 4 was brought abou either by treatment with chondroitin sulfat at pH 4, representing an electrostatic bind ing *of the positive groups of protein* to th

negative groups of chondroitin sulfate, or by preliminary alkali treatment at pH 7.6, both procedures followed by $CaCl_2$ treatment.[67] In both cases we propose that a critical configuration of negative charges is restored. While in the test tube the calcifying form of the protein molecule (almost certainly collagen) can probably be restored by several means, it is our belief that in vivo this is probably accomplished by some sulfated mucopolysaccharide or mucoprotein. Evidence in favor of this concept is the increased metachromasia in the presence of calcium ions[14,25,63] and restoration of calcifiability of demineralized bones with chondroitin sulfate.[67] Histochemical evidence from other laboratories is also in harmony with this concept.[9,29,31,36,39] We envision the action of the mucopolysaccharide or mucoprotein[25] as enhancing the calcium ion binding properties of the calcifying protein. The total calcium binding capacity of the cartilage would remain the same, since the chondroitin sulfate, by binding positively charged protein groups, endows the protein with a net negative charge equal to that of the original chondroitin sulfate. The additional function of the mucopolysaccharide sulfate as a calcium-concentrating vehicle, as proposed by Bélanger and Migicovsky,[10] is not excluded by this concept.

It must be borne in mind that $CaCl_2$ treatment, referred to in the foregoing paragraph, restores calcifiability to the acid-treated preosseous cartilage only in the normally calcifying portion. The proliferating zone of the preosseous cartilage does combine with calcium,[54] but this treatment does not induce subsequent calcifiability. It must be added that EDTA-demineralized bone treated only with $CaCl_2$ will not mineralize in vitro,[67] although it does combine with calcium, as shown by the fact that following treatment with high concentrations of phosphate, which produce nuclei, calcification will proceed both in the proliferating and the hypertrophic epiphysial cartilage.[54] It is for these reasons that we propose that activation by $CaCl_2$ depends on an active form of a specific protein molecule, probably collagen, that has innate properties for inducing nucleation.

Known Factors in the Nucleating System

It is evident from the foregoing discussion that the mineralization of collagen is only a limited picture of the system present in vivo. Further study by means of in vitro reconstitution of proposed models, as well as by extended studies of the actual living system, is needed to elucidate the mechanism responsible for nuclei formation in the intact animal.

The present state of knowledge of the factors involved in nucleation of collagen, as well as of bone, based on the evidence given earlier, is summarized below:

A. Evidence That Collagen Induces Nucleation

1. Reconstituted acid-soluble collagen induces mineralization.
2. Acid-insoluble collagen of bone matrix and from Achilles tendon also induces mineralization.[6] The mineralization in the matrix of bone and preosseous cartilage occurs in special areas that normally calcify, implying either that a specific form of collagen is involved[67] or that noncalcifying areas contain an inhibitory system.

B. Evidence Suggesting That the Nucleating System Contains Factors in Addition to Collagen

In vitro calcification of hypertrophic epiphysial cartilage takes place at a lower product of Ca × P and proceeds more rapidly than the in vitro mineralization of collagen.[6,29,61]

C. Evidence of Involvement of Mucopolysaccharides in Calcification

1. Chondroitin sulfate restores calcifiability in demineralized bone.
2. Gamma metachromasia appears in hypertrophic cartilage when it becomes cal-

cifiable. Dosages of x-ray that cause the disappearance of this gamma metachromasia result in a disappearance of calcifiability.[29]

3. Histochemical studies show the occurrence in the calcifying matrix of metachromatically active substances immediately prior to the onset of calcification.[9,31,36,39]

4. Both metachromatic and nucleating ability disappear in bones that have been frozen.[38] Both these properties reappear after treatment with calcium chloride.

5. Metachromasia in preosseous cartilage is enhanced by calcium ions. Such metachromasia, induced by pure chondroitin sulfate and other metachromatically active polyelectrolytes, disappears in the presence of calcium ions. This implies combination of some or all of the metachromatically active substance with a protein, probably collagen.[58,63]

D. Evidence for Other Likely Components of the Nucleating System

1. Presence of phosphatase in calcifying matrix.

2. Presence of enzymes of phosphorylative glycogenolysis.

3. Presence of enzymes of citric acid cycle.

4. Presence of energizing compounds, such as ATP and UTP, which are also sources of phosphate.

5 The probable existence of a so far undefined calcium-concentrating system.

E. Specificity of Nucleating Mechanism

1. Specificity of the nuclei-inducing collagen matrix is indicated by (a) the highly localized nature of normal mineralization, *although collagen predominates in other connective* tissues that do not mineralize; (b) proliferating epiphysial cartilage of bone (which also contains collagen) that does not mineralize even after restoration of mineralizability in demineralized rachitic tibial sections.

2. Specificity of action of the nucleating system is indicated by the inability to form nuclei with strontium, although strontium can contribute to crystal growth on calcium-containing nuclei.[18]

F. Evidence Suggesting Two Sites or a Cluster-Capturing Single Site in the Active Centers of the Nucleating Molecule

1. Either calcium or phosphate ions transform nonstriated collagen to collagen striated with a periodicity of 640 A.[4,62]

2. Cation linked inhibition of calcification in vitro by fluoride, cyanide and iodoacetate ions.[21,23]

G. Evidence for Activation of the Nucleating System of Preosseous Cartilage by Calcium Ions

1. Reversible inactivation of calcification in vitro of hypertrophic epiphysial cartilage was demonstrated.

2. Restoration of calcifiability in demineralized preosseous cartilage requires Ca ions. Mineralizability of demineralized true bone matrix is partially restored without calcium activation. Collagen, both acid soluable and acid insoluble, does not require calcium activation for its mineralization.

3. Ca ions prolong the survival of the calcifying mechanism of hypertrophic epiphysial cartilage.[22]

H. Evidence for the Probable Need of an Increase in Metastability $[(A_{Ca}^{++})(A_{HPO_4}^{--})]$ for Nucleation

Body fluids appear to be in equilibrium with bone crystals in vivo. This appears anomalous in view of the high degree of supersaturation of extracellular fluids with respect to bone mineral at a pH of 7.4. To *explain this discrepancy, the* formation of un-ionized complexes,[45] as well as a decrease in pH, has been suggested.[42]

At present, the minimum $(A_{Ca}^{++})(A_{HPO_4}^{--})$ product required for nuclei formation is not known. One can safely suggest that this is above that of the solubility of mature bone crystals. Thus, if body fluids are in equilibrium with the bone bed, then there should

be a need for locally increasing the concentration of Ca × P above the normal levels. It is conceivable, therefore, that the Ca × P product of body fluids is undersaturated with respect to the Ca × P product required for nuclei formation.

In all circumstances an increase in metastability would increase the rate of nucleation.

NUCLEI FORMATION IN BONE; A PROPOSED MECHANISM

We should like to offer some ideas concerning the nature of the nuclei-forming mechanism in collagen and to develop a scheme for the more complex system found in bone, the organic matrix of which is predominantly acid-insoluble collagen. It is visualized that collagen mineralization takes place either (1) when a prenucleus cluster formed in solution is captured as such by appropriately shaped active centers of the collagen fibrils, or (2) when critical negative charges on the collagen molecule bind calcium ions, and critical positive charges bind phosphate ions, creating a caged effect, enhancing the probability that these captured ions will interact to form a prenucleus cluster.

After the nucleus is formed it is ready for further growth in a supersaturated solution.

If the first mechanism operates, the capture of the critical cluster of calcium and phosphate by the matrix may occur either (a) at one site where the capture of the prenucleus cluster depends on the net charge of the microaggregate or (b) at two oppositely charged sites where capture of the prenucleus cluster of ions would depend on a critical distribution of positive and negative charges on the microaggregate.

If the second mechanism operates, the distance between the ion-capturing groups—in this case groups capturing calcium and phosphate—is critical. If the groups are too far apart, collision of the captured ions cannot occur, and, if they are too close together, it would be unfavorable for bringing about the right configuration of the cluster. It is conceivable that some form of energy plays a part in bringing about the optimal distance between the capturing groups.

From these considerations and other studies of calcification in vitro and in vivo, we visualize the nucleating mechanism in vivo as follows.

The nucleating macromolecule consists of a special form of acid-insoluble collagen. The nucleating structure is brought about by interaction with a sulfated mucopolysaccharide or mucoprotein and, in hypertrophic cartilage, requires activation by calcium ions. The active center of nucleation is visualized as a special arrangement of groups between the protofibrils of collagen. Nucleation with calcium and lack of nucleation with strontium indicate that a lock-and-key mechanism operates in inducing the formation of nuclei. A prenucleus cluster of calcium and phosphate ions is captured at the active center by one or two active groups, depending on the charge distribution on the cluster. The captured cluster then becomes a nucleus of crystal growth in situ.

The formation of nuclei is favored by an increase in the Ca × P product and an energy input. Vitamin D provides a systemic increase in the extracellular Ca × P product, particularly in the young. Components of the nucleating system, present in the calcifying matrix, which may provide energy and/or concentrate the ionic species, are (1) phosphatase; (2) the enzymes of phosphorylative glycogenolysis: (3) citric acid cycle; and (4) energizing compounds such as ATP and UTP. In addition, we propose a calcium-concentrating system, so far undefined.

SUMMARY

Nuclei formation depends on a metastable liquid phase with respect to the initial solid and is expedited by catalysts. Nucleation in mineralizing tissues requires a minimal product of calcium and phosphate ions in solution influenced by "local factors" in the organic matrix. Magnesium-linked inhi-

bition of calcification in vitro by anions and transformation of nonstriated collagen fibers to the 640 A spaced form by calcium or phosphate suggest two sites in the nuclei-forming macromolecule or a cluster-capturing single site. "Nucleated" rachitic sections calcify in vitro under conditions in which nonnucleated sections do not. "Nucleated" sections strontify in contrast with nonnucleated sections, indicating greater specificity for calcium in nucleation than in nuclei growth. Demineralized bone remineralizes following treatment with chondroitin sulfate or at pH 7.6 to 8.2, and is enhanced by $CaCl_2$ treatment. Available evidence suggests that, although collagen induces nucleation, the complete system is more complex, and probably includes (1) a specific form of acid-insoluble collagen; (2) sulfated mucopolysaccharide or mucoprotein; (3) enzyme systems such as glycolytic or citric acid cycles; (4) energy sources such as ATP and/or UTP; (5) a system concentrating calcium and phosphate ions. The active nucleating center is visualized as a site or adjacent sites, between the protofibrils of collagen, capturing a cluster of calcium and phosphate ions. The active center operates via a lock-and-key mechanism and is specific for conversion of the captured cluster of ions to a nucleus.

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Le Mechanismos Nucleoformatori in le Mineralisation Tissutal

Summario in Interlingua

Le formation de nucleos depende del phase liquide metastabile con respecto al solido initial. Illo es accelerate per catalystas. In tissus sub mineralisation, le nucleation require un producto minimal de iones de calcium e phosphato, influentiate per "factores local" in le matrice organic. Le facto que certe aniones, in le presentia de magnesium, inhibi le calcification in vitro e le facto que non-striate fibras de collageno es transformate per calcium o phosphato in le forma a spatios de 640 A suggere que il existe in le macromolecula nucleoformative (1) duo sitos critic o (2) un sol tal sito que es capace a capturar un bouquet de iones. Sectiones de tissu rachitic con nucleation es capace de calcification in vitro sub conditiones que supprime le calcification in sectiones rachitic sin nucleation. Sectiones a nucleation manifesta strontification per contrasto con sectiones sin nucleation que non manifesta strontification. Isto indica un plus forte specificitate pro calcium durante le processo del nucleation que durante le crescentia del nucleos. Osso dismineralisate manifesta remineralisation post tractamento con sulfato de chondroitina o a pH 7,6 a 8,2, providite que iste factores es sequite per tractamento a $CaCl_2$. Le informationes que es currentemente disponibile indica que, ben que collageno induce nucleation, le systema complete es plus complexe. Il es probabile que illo include (1) un forma specific de collageno que es insolubile in acido, (2) mucopolysaccharido o mucoproteina a sulfato, (3) systemas enzymatic, como per exemplo cyclos de acido citric o glycolytic, (4) fontes de energia, como triphosphato de adenosina e/o triphosphato de uridina, (5) un systema que effectua le concentration del iones de calcium e de phosphato. Le active centro de nucleation es visualisate como un sito o sitos adjacente inter le protofibrillas de collageno, capturante un bouquet de iones de calcium e de phosphato. Le centro active functiona per medio de un mechanismo a "serratura e clave" e es specific pro le conversion del capturate bouquet de iones in un nucleo.

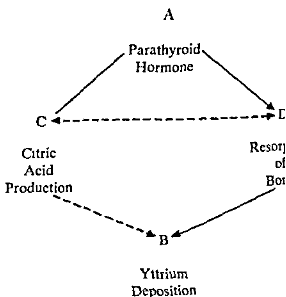
12

A Chemical View of Osteoclasis Based oı Studies With Yttrium*

W. F. NEUMAN, PH.D., B. J. MULRYAN AND G. R. MARTIN†

As recent publications attest,[12,19,2,11] it has been necessary to rely principally on morphologic evidence for our present concepts of the role of the osteoclast in the resorption of bone. Despite great progress, it is quite impossible to deduce, from purely structural considerations, the nature and the sequence of events at the molecular level. In most instances, chemical data have been distantly related to the events described by the morphologists and provide at best a shaky springboard for speculation. This has been unfortunate, for it is only in areas in which several disciplines converge that real understanding can be achieved.

A new chemical approach was made possible by recent advances in our understanding of parathyroid function. It has been shown that parathyroid hormone causes cells in bone to produce citric acid (possibly also lactate and other acids) and, further, that citric acid production causes an increased solubility of bone mineral locally.[23,9,21,24] It was tempting to conclude from this that acid production was a primary function of the osteoclast, since parathyroid hormone is a classic means of inducing the cellular resorption of bone. Though tempting, this conclusion is not warranted. Some further connection between the actual resorption prc and cellular production of acid is nee Accordingly, a study has been made of mechanisms underlying the skeletal de tion of radioactive yttrium. This eler has been shown to deposit preferentiall resorption cavities.[17] If a connection tween citric acid production locally yttrium deposition could be found, tempting conclusion would be validatec a kind of *overlapping triangulation of lo*



Known: That A induces C; also, D duces B and A induces D.

It follows: That if C also induces B, C an are closely interrelated proces

Experiments in vitro first demonstra that the mineral crystals of bone were sponsible for the remarkable affinity of

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skeleton for yttrium in confirmation of a very recent report.[14] *Since this finding could* not account in any way for yttrium's preferential deposition in resorption cavities, attention was directed to the diffusibility of yttrium in various solutions. When it was discovered that only a small fraction of yttrium could be ultrafiltered from normal serum and that the degree of ultrafilterability was greatly increased by elevated concentrations of citrate and hydrogen ions, the overlapping triangles of associated events were closed.

EXPERIMENTAL

The Filterability of Yttrium in Incubating Solutions

Yttrium is one of the rare earths that characteristically is insoluble, because it hydrolyzes at near-neutral pH to form a radiocolloid.[32,31,43] Such suspensoids tend to adsorb on containers, filters and solids, in general *rendering the study of these substances*, under physiologic conditions, difficult in the extreme. It is also characteristic of these rare earths to form stable, soluble complex ions in the presence of citrate ions.[31] For this reason, the solutions used for equilibrations had the following composition initially: 0.15 M KCl, 0.02 M diethyl barbituric acid, 0.002 M citric acid, the pH being adjusted to 7.4 ∓ 0.1 with saturated CO_2-free KOH.

To 1 L. of this solution, 1 Gm. of a synthetic hydroxyapatite[23] was added, and the suspension was stirred for 24 hours. Then the solution was rendered crystal free by filtration through millipore membranes (H. A. Whiteplain, Millipore Filter Corp., Watertown 72, Mass.), *and to the filtrate a tiny* aliquot of Y^{91} (Oak Ridge National Laboratory, Y-91-P) was added with vigorous stirring *An identical aliquot was added to* a similar volume of 2N HCl to serve as a colloid-free control solution At intervals, aliquots *of these 2 solutions* were passed through millipore filters prior to radioactive assay with a conventional thin-window Geiger-Müller tube and scaler. Expressed as per cent of the control valve (HCl solution) 92, 102, 106 and 102 were observed in the aliquots taken at 4 minutes and 2, 1 and 4 hours, respectively. Clearly, the small amount of citrate present in the incubating fluid prevented the formation of nonfilterable or easily adsorbed colloids. It must be remembered, too, that much of the citrate present had been removed by the initial incubation with apatite crystals.[1,9,35]

A second experiment was performed, with the same outcome. In this instance, the equilibrating solution, after prior exposure to apatite crystals, was divided into 5 100-ml. portions to which 50 λ aliquots of radioyttrium was added. Five controls (2N HCl) were prepared simultaneously. After standing for 2 hours at room temperature and filtration, the average recovery, *in per* cent of controls, was 101 ∓ 4.

Table 1. The Failure of Bone Matrix To Bind Yttrium

Experiment	1	2
Weight of bone matrix (Gm.)	0.132	0.108
Yttrium content in solution at:	%	%
5 min.	99	99
30 min.	102	101
1 hr.	100	100
24 hrs.	99	99

The Failure of Bone Matrix To Bind Yttrium

Bits of fresh human cortical bone were *demineralized exhaustively* with neutral solutions of ethylenediaminetetraacetic acid, as described elsewhere.[36] Portions of this *demineralized* bone were placed in 100-ml. portions of the equilibrating fluid that had had a 24-hour prior exposure to apatite crystals followed by the addition of radioyttrium. At various time intervals during stirring, aliquots of the solution were taken for radioactivity assay. The results, given in Table 1, show conclusively that bone "ma-

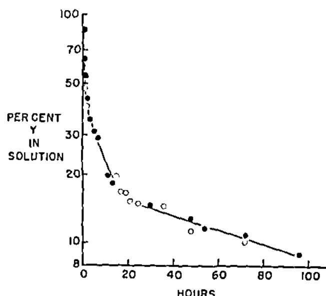


FIG. 1. The disappearance of yttrium from solution as function of time as the element enters the crystalline phase. Conditions: 25° C.; 1 liter of solution; μ = 0.17, pH, 7.4, 0.1 Gm. hydroxy apatite. Solid and open circles represent data from separate experiments.

trix" does not adsorb or bind significant quantities of yttrium.

It cannot be claimed that *only* inorganic material is removed by the demineralization process. Certainly, most of the collagen formed and much of the polymerized mucopolysaccharides remain in the tissue residue. Nonetheless, it is possible that some organic substance with a specific affinity for yttrium was dissolved selectively during demineralization. However, as shown below, it is unlikely that such a substance, *if it exists,* could compete significantly with the bone mineral itself for yttrium.

The Exchange-Adsorption of Yttrium by Calcium Phosphate

To 1-L portions of the equilibration solution various amounts of hydroxyapatite crystals were added, and, after 24 hours' equilibration at 25° C., 0.1-ml. aliquots of radioyttrium were added. Thereafter, at varying time intervals, aliquots of the suspension were removed and filtered, and the crystal-free filtrate was assayed for radioactivity. These results are assembled in Table 2.

TABLE 2. THE EXCHANGE-ADSORPTION OF YTTRIUM BY HYDROXYAPATITE CRYSTALS

Time After Y Addition	Per Cent Y Left in Solution			
10 min.	81	34	5.6	<1
1 hr.	56	14	2.2	<1
2 hrs.	39	10	1.2	<1
Solid: Solution, Gm./L.	0.1	0 3	1.0	2.0

The crystals were extraordinarily effective in removing yttrium from solution. For example, at a solid-to-solution ratio of 1:1000, a ratio at which bone "matrix" removed no measurable quantity of yttrium, the crystals removed 99 per cent in 2 hours! Compared with other elements studied in this laboratory (Ca^{45}, Ra, Sr-89, UO_2 and $P^{32}O_4$), yttrium's affinity for hydroxyapatite is greater by orders of magnitude.

For further study, a solid-to-solution ratio of 0.1 Gm. per L was selected. The time course of the reaction was examined in detail and is presented in Figure 1. Unlike the kinetics of the exchange of alkaline earths, Ca^{45},[Ref 42] Sr-89[Ref 13] and Ra,[Ref 26] the curve is continuous and cannot be separated easily into 3 individual reactions. Whether this represents a new *kind* of exchange or whether the rate-limiting step involves a change in the state of yttrium *prior* to its exchange is not yet clear.

That some kind of exchange reaction is involved is indicated by the sensitivity of the process to changes in calcium concentration in the equilibrating fluid. These variations were accomplished by an addition of small amounts of $CaCl_2$ to the equilibration fluid 24 hours prior to the addition of the yttrium. Calcium concentrations in the solutions were determined[40] at the conclusion of the experiment. These data are given in Figure 2. Because no steady state or quasi equilibrium was achieved, the conventional application

of mass law to ascertain the mole ratio of the exchange of Y for Ca[13,26] is not permissible.

Attempted Reversal of Yttrium Exchange

In previous studies[13,26] the addition of calcium ions midway in the course of a radiocation exchange caused a sharp reversal, the radioisotope returning to solution from its deposition sites on the crystal's surfaces. Accordingly, to 1 L. of the equilibration solution 0.1 Gm. of hydroxyapatite crystals was added, and, after being stirred for 24 hours at 25° C., 0.1 ml. of radioyttrium was added. The course of the exchange was followed as before by removing aliquots at various time intervals and performing radioactivity analyses on the crystal-free solutions after filtration through millipore membranes. Three hours after addition of the radioyttrium a small volume of equilibrating fluid containing 30 mg. of Ca as $CaCl_2$ was added. Then the course of the exchange was followed for an additional 3 hours. These data, given in Figure 3, do not show a sharp reversal on the addition of calcium ions; rather, yttrium's affinity for the mineral phase was so great that the course of the exchange was "interrupted" only temporarily by the sudden rise in calcium ion concentration. Clearly, yttrium is in sharp contradistinction to the alkaline earths as regards the avidity with which it seeks lattice sites.

Despite some uncertainties as to the *nature* of the exchange process, there seems to be little question that it provides an adequate explanation of yttrium's pronounced tendency to concentrate in the skeleton. While these studies were in progress, a report appeared[14] describing a slightly different experimental approach that led to the same conclusion: it is bone mineral rather than bone matrix that attracts yttrium. Therefore, the experiments just described must be regarded as confirmatory of an already established view.

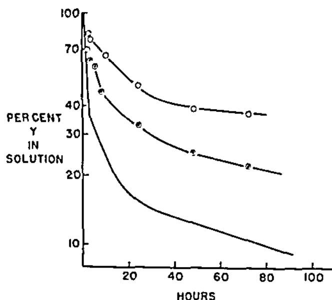


Fig. 2. The effect of calcium concentration on the course of the yttrium exchange. Conditions were as in Figure 1, and the solid curve is taken from that figure Dotted circles were data obtained when 30 γ ml./Ca^{++} was added 24 hours prior to introduction of yttrium; undotted circles, data from the addition of 60 γ ml /Ca^{++}.

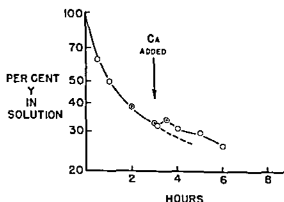


Fig. 3. An attempt to reverse the exchange of yttrium by the addition of calcium, 30 γ/ml., at 3 hours. In all other respects conditions were as given in Figure 1.

The fantastic affinity of apatite crystals and, therefore, of bone mineral[14,27] for yttrium furnishes ample reason for classifying yttrium as a "bone-seeking" fission product.[18,19] However, the fact that it is the bone mineral, rather than the organic matrix, which binds the yttrium would lead one to

expect that the histologic distribution of this element in vivo would correspond to the distribution of other bone-seeking cations, such as Ca^{45}, Sr^{90}, Ra, etc. These latter radioelements have a strong affinity for bone mineral and deposit preferentially in those skeletal areas in which the crystals are easily accessible by diffusion—the areas of new bone formation that have not fully mineralized.[27] Yttrium, however, exhibits a different pattern of deposition in vivo. Some, it is true, is found in areas of new growth (or so it was interpreted in early work[16,29]), but the unique aspect is yttrium's proclivity for resorption cavities, the surfaces of bone under cellular resorptive attack.[14,15] Since this puzzling difference in yttrium's physiologic behavior could not be explained in terms of its affinities for a bone substance, the answer to the paradox was sought in terms of transport.

The alkaline earths Ca, Sr and Ra do not hydrolyze at physiologic pH and are, for the most part, ionic and diffusible, only about a third being bound to protein.[13,26,41] The level of these substances, then, in the total extracellular fluids can be expected to be about two thirds of the blood level and equilibrating rapidly with the circulation. The alkaline earths can reach all parts of the skeleton freely, and it is the state of the skeleton itself, its degree of hydration, that determines the pattern of the exchange-deposition.[27] Yttrium, on the other hand, hydrolyzes to form radiocolloids at neutral pH.[32,31,43] Its level in the extracellular fluids might be expected to be much lower than that in the blood, and the transport of yttrium between the 2 compartments is difficult to predict.[32]

The Lack of Diffusibility of Yttrium Under Serum Conditions and the Effects of pH and Citrate

From these considerations attention was turned to a study of the diffusibility of yttrium under a variety of conditions approximating physiologic.

For the first experiment, 43 ml. of serum was obtained from freshly drawn blood from the jugular vein of the dog. Ten ml. of serum was reserved, and the remaining 33 ml. was equilibrated with 5 per cent CO_2 and ultrafiltered at 37 to 41° C.[Ref 39] until more than 10 ml. of clear ultrafiltrate was obtained. At this point, to both the original serum and the ultrafiltrate, small identical aliquots (0.2 ml.) of radioyttrium were added, and, after equilibration with CO_2, the samples were ultrafiltered at 39° C. The concentration of yttrium in the ultrafiltrate of serum, as determined by radioactivity, was only 0.5 per cent of the level in the serum. Even in the absence of serum protein (in serum ultrafiltrate), only 5 per cent was ultrafilterable (cf. Ref. 31). In one instance, the apparatus developed a gas leak, permitting the escape of CO_2, which caused the pH to rise to 8.0. In this case, despite the absence of protein, less than 0.1 per cent passed the membrane.

The results confirmed the fact that yttrium forms a colloid under physiologic conditions[32,31] and established further that there exists in blood no complexing substances in sufficient concentration to render the yttrium freely diffusible. From the present data it cannot be said that proteins *bind* yttrium (cf. Ref. 31), but they and pH have an important influence on its diffusibility.

Previous experience in our laboratory with other hydrolyzable elements (UO_2,[Ref 6] Be[Ref 8] and Po[Ref 7]) has been most discouraging. Years of effort are required to obtain reproducible systems, and even then it is questionable whether or not the data obtained from such rigorously controlled laboratory conditions can be generalized. For this reason only qualitative relations were sought. Approximately 20 μc. of radioyttrium was administered intraperitoneally to each of 10 rats, and, after 1½ hours, blood was drawn by heart puncture from the animals under light ether anesthesia. The collected blood was pooled, allowed to clot and centrifuged. The resulting serum

TABLE 3. THE EFFECT OF pH AND CITRATE ON THE ULTRAFILTERABILITY OF Y FROM SERUM

Serum Sample	Experiment 1		Experiment 2	
	Y Ultrafiltered	Citrate Content	Y Ultrafiltered	Citrate Content
	%	mg. %	%	mg. %
Control 5% CO_2	4.2	2.8	0.3	5.0
Control 100% CO_2 pH 6.0	12.0	3.0	15.0	4.6
Citrate added, 5% CO_2	21.0	9.8	2.3	7.9
Citrate added, 5% CO_2	36.0	19.0	6.8	11.0
Citrate added, 5% CO_2	—	—	7.0	17.0
Citrate added, 5% CO_2	—	—	9.6	16.0

was divided into portions. One portion was equilibrated with 5 per cent CO_2, another was equilibrated with 100 per cent CO_2, while others received tiny aliquots of sodium citrate solution before equilibration with 5 per cent CO_2. All then were ultrafiltered at 38° C. Radioactivity assays and citrate analyses[3] were performed on all specimens and their corresponding ultrafiltrates. The results of 2 such experiments are assembled in Table 3.

As expected from the previous experiments in vitro, only a small variable fraction of yttrium was ultrafiltered from normal serum. Also, as expected, lowering the pH to 6.0 greatly increased the fraction ultrafiltered. Of great interest—and not wholly unexpected—was the finding that small amounts of citrate also greatly increased the fraction of yttrium ultrafiltered from the serum.

The variability inherent in such an unstable colloidal system leaves much to be desired. To give added weight to the data thus far reported, 2 further experiments were performed. In one case, a solution of yttrium in perchloric acid was titrated with alkali to determine at which pH hydrolysis occurs. In the second instance, radioyttrium was added to 2 samples of bovine blood in order to provide larger samples of serum for ultrafiltration experiments.

The results of the titration are given in Figure 4, two features of which are note-

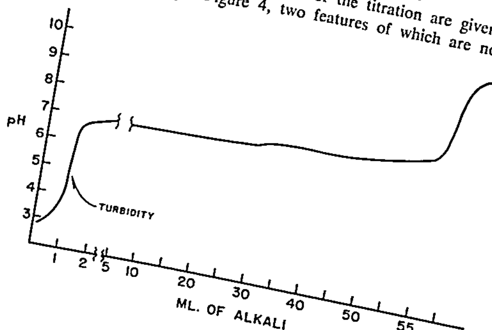


FIG. 4. The titration of 0.1 N yttrium chloride with alkali. $Y(NO_3)_3$ was dissolved in 0.05 N $HClO_4$ (to prevent spontaneous hydrolysis), and 50 ml. was titrated with 0.0964 N NaOH. Plotted in the graph are the milliliters of alkali in excess of that needed to neutralize the perchloric acid.

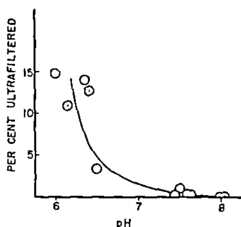


FIG. 5. The effect of pH on the ultrafilterability of yttrium added to bovine serum. pH was varied by varying the partial pressure of CO_2. Though two different samples of bovine sera were used, no clear differences between them were seen.

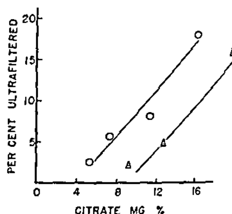


FIG. 6. The effect of added citrate on the ultrafilterability of yttrium. Here, the two samples of bovine sera responded differently, though, qualitatively, increasing citrate concentrations increased the ultrafilterability of yttrium in all instances

worthy. First, significant buffering (which signifies hydrolysis) occurred just around the physiologic range! This suggests that below pH 6 yttrium is largely ionic, while above pH 7.4 it is almost completely hydrolyzed. Second, the number of equivalents of alkali used in the hydrolysis was greater than 1, a result characteristic of polymerizing systems. To solve even partially the physiochemical events occurring in such a complex hydrolysis, years of study frequently are required. However, for present purposes it was deemed necessary to establish only the pH range at which the hydrolysis occurred.

The ultrafiltration data have been assembled in Figures 5 and 6. Small aliquots of a strong solution of citrate were added to some samples, while others were equilibrated with varying pressures of CO_2 to vary the pH. As before, both citrate concentration and pH influenced markedly the diffusibility of yttrium.

Though no direct evidence is as yet available, one would expect that hydrogen ions and citrate ions would be additive in their effects, at least at pH 6 or above Below pH 6.0 citrate becomes less ionized,[20] and its chelating powers are thereby diminished.

At the moment, it is difficult even to guess at the levels of citrate in histologically defined areas of bone. It has been estimated that, under a parathyroid stimulus, the *average* citrate level of venous outflow from bone is over 8 mg. %.[21] Since this average represents a mixture of blood from areas of forming as well as of resorbing bone, it is possible that the citrate levels in resorbing sites are several times greater than this average, as great or possibly greater than the concentrations employed in the present experiments.

For these reasons it is not unrealistic to conclude tentatively that yttrium *would* show a distribution in bone similar to that of the alkaline earths if it were freely diffusible. Because it is not, it can reach bone mineral best in those sites in which the hydrogen ion and citrate ion concentrations become elevated in the capillaries as the result of localized production of these ions by cells. On this basis yttrium shows promise, as a histologic marker, of citrate and/or acid production in bone.

Since the parathyroid hormone has been linked to citric acid production in bone as well as to osteoclasis of bone, and since yttrium seems to require citric acid production

for transport to bone and deposits preferentially in sites of osteoclasis, it follows that osteoclasis involves the production of citric acid. The case is not proven, but, until contrary evidence is forthcoming, it remains an attractive hypothesis for designing future experimentation.

DISCUSSION

Since 1873[17] osteoclasts have been associated with the resorption of bone. There is no question of the ability of osteoclasts to resorb bone, but there has been considerable uncertainty as to the means by which this is accomplished.[12,19] There is also doubt as to the origin and the fate of these fascinating multinucleated cells, and it is not known whether or not their presence is required for the dissolution of bone substance in *all* instances.[12,19] In brief, the processes of bone resorption are poorly understood.

From the chemical viewpoint it is much too early to speculate profitably on what events might induce the formation and the disappearance of the osteoclast. However, there is a growing body of experimental evidence that by exclusion renders unlikely some of the conjectural suggestions concerning the mechanisms of osteoclasis. The question as to whether or not cells are necessary for bone dissolution can also be answered, albeit tentatively.

At present it seems most likely that bone substance, an extracellular mass of interwoven fibers, mucoid and inorganic crystals, requires cellular activity for its removal. The most powerful argument in favor of this view is the low content of water found in established bony structures. It has been shown that all but a few per cent of the total water present in formed bone can be attributed to perivascular and pericellular spaces, leaving very little free water available for the intercellular bone substance.[30] It can be calculated that the free water spaces between the microstructural elements (crystals and fibers) are so tiny that *free* diffusion of even ionic substances is quite impossible.[27] In support of this conclusion it has been observed that even a small univalent ion, such as sodium, and ion limited to the bound ion layer at the surfaces of the tiny crystallites in bone[34,35] can diffuse freely into only a small fraction of the adult skeleton.[27]

If ions cannot diffuse freely *in*, how can bone dissolve passively by the diffusion of ions *out*?

Even if the problem of diffusion is ignored, it has been established that the body fluids normally are quite supersaturated with respect to bone mineral,[27,36] a fact that argues against the passive dissolution of a single crystal of bone mineral even if the dissolved ions could diffuse away from the site.

Continuing with the argument, should the problem of diffusion and the problem of supersaturation be disregarded, there still remains the organic matrix, of which some 90 per cent is collagen. As proteins, the collagens rank among the most stable of all, both chemically and physiologically. The physiologic turnover of bone collagen is extremely slow,[33] and, as a class, the collagens can be dissolved only in strongly acid or alkaline solutions. In our experience, bone collagen does not dissolve even in the acetic acid solutions used to solubilize collagen derived from connective tissues.[36]

On chemical grounds, then, the evidence is almost overwhelming that active, energy-expending cellular activity is required to remove bony substance once it has formed. The question remains, what is the order of events? Is the mineral removed first, exposing the matrix to enzymatic attack, or is an enzymic induced change in the matrix required to "release" the mineral, as has been suggested frequently?[12] To answer this question it is necessary to return to the problem of limited diffusion in bone. Obviously, if sodium ion cannot diffuse into the bulk of established bone, neither can complex protein molecules, such as proteolytic enzymes, diffuse into bone to attack the matrix. This conclusion has been confirmed

by direct experimentation in 2 laboratories.[10,22] Dead, defatted powdered bone was exposed to several kinds of proteolytic enzymes (trypsin chymotrypsin, papain, collagenase, etc.) both singly and in combination without a significant loss of nitrogen from the powder. If these incubations were carried out at low pH, or if the powder first was demineralized, all these enzymes were active in the proteolysis of the bone matrix.[22]

From these considerations one is led to the view that cells are required for the removal of bone and that this process must involve the active cellular secretion, first, of acids (or other solubilizers of bone mineral) and, second, of proteolytic enzymes. The reasonableness of this view is attested by 3 lines of investigation. First, the present studies with yttrium offer, not proof, but strong evidence that the cellular production of citric acid and resorption of bone are closely interrelated processes. On histochemical evidence, Cretin[4] also deduced that osteoclasts produce a localized lowering of the pH. Second, in tissue culture, active proteolysis by osteoclasts has been observed.[3] Third, the histologists find many morphologic characteristics strongly suggestive of secretory activity.[12]

All these arguments do not preclude the occurrence of phagocytosis as an important aspect of bone resorption. Nonetheless, it is ridiculous to suppose that an osteoclast could phagocytize an entire bone spicule as a piece or that an osteoclast could approach an endosteal surface and swallow it whole. If the diffusion argument has any merit at all, it is this: phagocytosis can be only a process that is secondary to acid and proteolytic attack, the pseudopodic envelopment of bits and pieces that have crumbled from structures weakened by prior digestion. Perhaps this accounts for the rarity with which osteoclasts containing bony substances are seen [3,13]

In summary, one can still say that the processes of bone resorption are poorly understood. However, on chemical grounds only a few possibilities seem reasonable. Better still, these possibilities are amenable to experimental attack, especially with new technics,[11,28] a fact that augurs well indeed for the future.

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Le Problema del Osteoclase ab un Puncto de Vista Chimic, Super le Base de Studios con Yttrium

Summario in Interlingua

Durante que le terras alcalin se depone preferentialmente in elementos del skeleto currente- o recentemente in formation, studios histologic ha demonstrate que yttrium radioactive se concentra preferentialmente in sitos skeletic que se trova in statos de resorption active. Pro explicar le comportamento paradoxe de yttrium, le essayo esseva facite de elucidar le mechanismos per que iste terra rar es fixate in osso.

Il esseva trovate in vitro que yttrium se combina avidemente con le substantias mineral del osso, sed illo non reageva quando illo esseva exponite al portion organic del osso (i.e. a preparatos dismineralisate per acido ethylenediamino-tetraacetic). In iste respecto, yttrium se comportava similemente al terras alcalin.

Per medio de technicas de titration e ultrafiltration il esseva demonstrate que yttrium experientiava hydrolyse e polymerisation in le region de pH 6 a 8, con le resultante formation de non-diffundibile colloides que esseva incapace de passar per membranas semipermeabile. In plus, il esseva constatate que seros normal contine nulle substantias complexante que es capace de render yttrium diffundibile. Quando le pH esseva reducite o quando citrato esseva addite, le colloides de yttrium se decomponeva al minus in parte de maniera que le metallo deveniva plus prestemente diffundibile.

Iste constatationes es indicios presumptive que le distribution distinctive de yttrium es le consequentia de conditiones de transporto, specificamente que yttrium trova facile quitar le capillares solmente in areas que es ric in citrato e/o altere acidos. Un plausibile consequentia de isto es que le sitos de resorption (in que yttrium se depone preferentialmente) as sitos de production de acido Le signification de iste intime association inter resorption e production de acido es discutite ab le puncto de vista del mechanismos chimic que representa le processo del osteoclase.

13

Isotope Studies of Bone Salts*

Marcel J. Dallemagne and Léon Richelle†

For 20 years radioisotopic technics have been utilized to obtain information concerning the molecular structure of bone salts. In reviewing the work accomplished during this period, three questions are in order:

1. How have the results already obtained with isotopes been interpreted?
2. In what chronologic and logical order was this work accomplished?
3. What are the possibilities offered by this method?

In order to state the problem clearly, we will summarize the present concept of bone and dental salts and of the synthetic phosphates that are similar chemically.

As accepted generally, bone salts are the inorganic elements of bone and teeth that can be obtained by boiling the material in glycerol containing 6 per cent KOH or by treatment with ethylenediamine. X-ray diffraction data indicate that the crystalline lattice of these salts is similar to that of hydroxyapatite. Accordingly, for the past 30 years bone and tooth salts have been referred to as hydroxyapatite. However, the biologic and the mineralogic salts are not absolutely alike, and we believe that the choice of the hydroxyapatite model is not suitable for the following reasons:

1. Bone and dentin contain more calcium than hydroxyapatite.
2. Bone and dentin contain a rather large number of CO_3 groups that are too large to substitute for the OH groups of hydroxyapatite.[23]
3. Bone and dental salts are hydrated, just as synthetic phosphates are. This is not so true of mineralogic hydroxyapatite.
4. Synthetic phosphates that present the lattice of hydroxyapatite can contain less, or more, calcium than the latter compound. Hydrated phosphocalcic compounds with the apatitic lattice may be considered as the members of a continuous series that contain the same number of phosphorus atoms but different quantities of calcium atoms.[12] Generally, bone salts contain 10.5 Ca for every 6 P atoms, this being equivalent to the highest Ca/P ratio of the series. A corresponding calcium phosphate may be synthesized. Conversely, octocalcium phosphate, which is the lowest member, contains only 8 calcium for every 6 phosphorus atoms.[3] Between these two extreme values, with Ca/P weight ratios of 2.26 and 1.72, respectively, all the intermediate values may also be obtained synthetically. Hydrated tricalcium phosphate corresponds to only one of them with an integer amount of calcium and phosphorus atoms.‡

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‡ It was demonstrated recently in our laboratory that hyd[illegible] properti[illegible] groups, [illegible] transformed into pyrophosphate groups The latter give specific bands on infrared spectrograms and may be estimated quantitatively by chemical methods Synthetic hydroxyapatite (Ca/P=2.14) does not contain such groups.[54] Preliminary studies have shown that pyrophosphate groups also are present in bone and dentin heated below 600°C This is the first direct proof that the main phosphate constituent of biologic hard tissues contains fewer Ca atoms than hydroxyapatite in spite of their high Ca/P weight ratio [25]

The evolution of the chemical composition of the members of this series may be interpreted in several ways. Among the natural or synthetic calcium phosphates, it must be stressed that the so-called hydrated hydroxyapatite with a Ca/P weight ratio of 2.14 is the most stable. Conversely, the lattice of the lowest members of the series is lacking in calcium ions.[47] (Some authors[15,37] claimed that H_3O groups were present in hydrated tricalcium phosphate. It has not been possible to detect such groups by means of infrared spectrography.[54]) When apatitic compounds of this kind are suspended in a lime solution, calcium ions replace hydrogen ions, and the Ca/P ratio increases.[18,19] This ability to replace hydrogen ions in the lattice with Ca ions constitutes the basis for "the defect compound theory."

Opposing this concept of a defect compound theory are the investigators who believe that all the members of the phosphocalcic series are composed of hydroxyapatite but that more or less PO_4 groups are adsorbed at the surface of the microcrystals.[31,17,52,24] The radioisotope of phosphorus was used for the first time by Manly and Levy[10] to study this latter concept. P^{32} was employed to detect the location and the movement of small amounts of phosphorus. They observed that bone powder, suspended in boiling ethylene-glycol containing KOH and tagged Na_2HPO_4, was able to fix P^{32} just as a solid would adsorb from a gaseous phase.

From 1939 to 1947 the scientists of the Rochester School of Medicine supported this opinion, and all the experiments were interpreted on this basis; i e., the natural and the synthetic phosphates are able to adsorb PO_4^{---} ions In 1940 Armstrong[2] also stressed the importance of the crystal surface. According to this author, bone, dentin and enamel salts do not fix the same amount of radioactive phosphorus because the crystal volume of these three tissues is not the sam consequently, the crystal surface is diff

On the other hand, by experiments formed in vivo, some authors established that radiophosphorus was not only adsorbed by the skeleton but might possibly exchange with the phosphorus already present in bone; moreover, physiologic remodeling of bone and accretion phenomena were able to induce the penetration of radiophosphorus in bone.[38,40,26,4,53]

It is interesting to note that in 1945 Johansson, Falkenheim and Hodge,[30] of Rochester, suggested that adsorption was probably not the only cause of the in vitro penetration of bone salts by P^{32}, that another mechanism was operating, but they were unable to define it. These authors did not readily accept the concept of exchange, because the amount of P^{32} that went into bone salts was too high. At that time biologists were not acquainted with the exchange concept that had been defined by Paneth in 1912. The exchange percentage measured at equilibrium corresponds to the distribution of an isotope between two phases It may be calculated from the specific activities ratio, i.e., the quotient of radioactivity/mg. of the considered element.*

The interpretation of Johansson *et al.* seemed to be influenced by their working hypothesis that bone salts were converted into tricalcium phosphate by PO_4^{---} ions adsorption.

In 1947 the concepts of the Rochester School were renewed, and Falkenheim, Neuman and Hodge[20] demonstrated that metabolic accretion was only a secondary process that occurred in bone to explain the penetration of P^{32} into this structure. These authors demonstrated, by chemical analysis and measures of radioactivity in both the bone salts and the liquid phase, that isoionic exchange, not adsorption, was the main mechanism of P^{32} penetration of bone salts. Indeed, they observed that the composition of the liquid phase ot vary when the

* This conception the exchange percentage measured at corresponds to the ibution of an is n two phases It ents the theor experiments persince 1947.

majority of the radioactivity of this phase was lost. The exchange reaction was reversible; i.e., after exchange, if the bone was immersed in a nontagged phosphate solution, the P^{32} was lost from the bone salts. From that time P^{32} has been used, both to detect small amounts of P and to study the mobility of the PO_4^{---} ions located at the surface of the microcrystals.

Hodge[28] stated in 1949 that isoionic exchange was the principal mechanism operating both in vitro and in vivo for the introduction of isotopes into bone salts or into the skeleton. Thereafter, Neuman *et al.*[42,44] specified that isoionic exchange of bone mineral should be considered from the point of view of surface chemistry. The huge surface of bone salt microcrystals (more than 100 sq. M. per Gm.) is the principal feature that regulates the ionic equilibrium of bone mineral with surrounding liquids This surface may be appreciated by means of P^{32} exchange reaction. The chemical character of this reaction depends on many factors—temperature, pH, etc. In order to obtain reproducible results, bone salts must be pretreated with a buffer solution that represents the eventual liquid phase.[41] As we shall see later, this precaution leads to the important fact that bone salts are not stable when suspended in water; thus the experiments of Neuman were made on already remodeled material. As soon as radiocalcium was available, Hodge, Falkenheim and Emery[29] repeated with this isotope the experiments already performed with P^{32}. A preliminary report on this topic appeared in 1947, a detailed one being published in 1951 by Falkenheim *et al.*[21] Calcium exchange of bone salts in vitro is also reversible, the calcium exchange percentage being higher than for phosphorus. These authors believed that this difference depended on the 0 5 supplementary calcium atom that bone salts contain in respect to the hydroxyapatite molecule. As is the case with phosphorus, calcium penetration of bone salts is a two-step reaction—a rapid phase corresponding to exchange and a slow one due to recrystallization. The same is true of dentin and enamel, although, for the mineral part of these two tissues, the exchange percentage is lower because of the greater dimension of the elementary crystals. In 1953 Neuman and Neuman published an interesting general review of the question.[45] They believed that for calcium, as for phosphorus, a great many points remained to be clarified regarding factors that would be able to influence the exchange reaction. Because of this, it does not seem possible to state quantitative deductions. However, it may be anticipated that such data will be available in the future.

In 1953, in Liège, we undertook experiments conducted in the following manner:

The basis of our first experiments was the work performed by Logan and Taylor in 1938.[36] These authors observed that it was possible, by using small amounts of HCl, to extract progressively from bone salts the excess calcium that they contain in respect to tricalcium phosphate. In this way it was possible to lower the Ca/P ratio of bone salts by successive acid washings. Thus it seemed that the binding energy of all calcium atoms of bone salts was not the same. These observations have since been verified many times by Cartier[6] and ourselves.[7]

Bone salts were suspended[10,15,16] and stirred in a $Ca^{45}Cl_2$ solution for 1 week; thereafter they were leached successively by dilute HCl samples. We observed that stoichiometrically each acid liquid phase extracted more calcium than phosphorus in respect to the composition of hydrated tricalcium phosphate; the specific activity of this calcium excess was the same after each hydrochloric acid attack. The interpretation seemed clearly to be the following:

The fraction of bone salt-calcium undergoing exchange was precisely the excess calcium that raised the Ca/P ratio of bone from 1 94 (tricalcium hydrated phosphate) to 2 26. But our results were not in agreement with the principle already propounded by

Neuman.[45] The radiocalcium that we found in bone salts was not located only at the surface but seemed to be dispersed inside the microcrystals. In some experiments performed with longer exchange times we even found more radiocalcium inside than at the surface. This discrepancy between Neuman's results and our own was clarified later.

Afterward we tried to measure the time necessary to obtain an equilibrium.[12,14] In our minds a true surface exchange phenomenon should be instantaneous. We observed repeatedly that the exchange reaction was complete after 20 hours and that approximately 14 per cent of the calcium of bone salts was exchanged. This value was related closely to that published previously by Hodge and collaborators.[21,29] On the other hand, another correlation was possible and confirmed a conclusion that previously had been presented; namely, that 14 per cent of the total calcium contents of bone corresponds to the excess calcium that this material contains in respect to hydrated tricalcium phosphate. It appeared likely that this excess calcium was the exchangeable calcium of bone salts, since it corresponded to the 1.5 Ca atoms of the total 10.5 Ca/6 P atoms that they contain (and perhaps to the calcium ions bound to CO_3^{--} groups[12]).

Some experiments performed thereafter on total bone gave our researches a new orientation[14] Total bone powder suspended in a $Ca^{45}Cl_2$ solution, and stirred until equilibrium was reached, resulted in a 24 per cent exchange after approximately 20 days. The time necessary to obtain equilibrium depended on the rate of diffusion of ions through the organic matter. The rate of exchange was closely temperature dependent, increasing linearly with temperature rise. We were surprised to find that total bone exchanged more calcium than bone salts and concluded from these experiments that mineralization by Gabriel's method or by ethylenediamine reduced the mobility of 1 calcium atom. In total bone, indeed, 2 5 atoms of the possible 10.5 Ca 6 P are exchangeable, instead of 1.5. A series of controls showed us that if total bone were boiled in anhydrous glycerol containing 6 per cent KOH and washed afterward with alcohol instead of water, the exchange reaction looked very different—about 24 per cent of the calcium atoms are exchangeable in a very short time (20 min.) This speed of reaction seemed to us to be consistent with the concept that exchange reaction takes place at the surface of the bone salt microcrystals. Moreover, this mineral, which had not been treated before the exchange reaction, exchanged the same amount as total bone.[12]

After these experiments the opinion concerning bone salt structure had to be revised. The phosphocalcic compound that represented the basis of bone salts was constituted by 8 Ca and 6 P atoms, i.e., octocalcium phosphate, as described by Arnold[3] In this condition, 2.5 Ca atoms are located at the surface of the microcrystals and are extremely mobile. The octocalcium phosphate is very unstable in aqueous medium and completes its internal defect structure at the expense of the surface calcium excess When included in the inorganic matrix, this compound is very stable because the defects of the lattice are completed electrostatically by organic radicals. These radicals are destroyed during mineralization and substituted by hydrogen ions, which, in turn, are easily substituted by Ca ions, when available.[8]

But the interpretation of the experimental data that leads to such a fascinating concept was no longer satisfactory after the following listed findings:

1. CALCIUM EXCHANGE OF TOTAL BONE

It is well known that bone slices suspended in a tagged calcium solution fix calcium.[1] The autoradiographic method makes possible a topographic study of the isotope deposition. Radiocalcium does not diffuse deeply into the slices and is deposited in

some poorly mineralized areas (hot spots), a fact that may be verified by microradiography. In these areas the organic material is hydrated, and diffusion is rather easy. In other words, the penetration of total bone by radioisotope is not homogenous. Thus, the 24 per cent exchange that we observed might represent only a mean value, being partially a true exchange (desorption in a $CaCl_2$ solution being possible) but mainly an adsorption, depending on the organic matrix (desorption occurring in distilled water). When the organic matrix is removed, the distribution of the isotope is homogenous, and hot spots no longer appear on the autoradiograph.[9]

It may be concluded that exchange reaction values assigned total bone are valueless when slices are concerned, and new investigations of total bone powders are necessary to confirm or reject preceding results as to their interpretation.

2. PEPTIZATION OF BONE MINERAL

The calculated exchange percentage value of 24 per cent observed in mineralized bone does not represent a true exchange reaction, but, as is stated later, this figure depends on several factors.[51]

The exchange percentage is the ratio between the specific activity of the solid phase ($\times$ 100) and the specific activity of the liquid phase at equilibrium. Stability of the calcium concentration in the liquid phase is essential in order to obtain a true exchange percentage value; however, this condition is never absolutely fulfilled. If mineralized bone (250 mg.) particles are stirred in a tagged $Ca^{45}Cl_2$ solution containing less than 200 μg Ca/15 ml., the solution contains more calcium after reaching equilibrium. On the other hand, if the liquid phase contains more calcium, then the solid fixes calcium Initially in our study the first-mentioned set of conditions appeared to be more desirable, as we thought that the distribution of the isotope could follow constantly the movements of calcium between the solid and the liquid phase so that the exchange equilibrium would be corrected immediately. Conversely, the use of a high calcium concentration in the liquid phase seemed to us an unrealistic condition, because very likely it corresponded to the penetration of the solid by isotope by a remodeling process that could be much easier in this latter condition.

Actually, when equilibrium is reached, if the exchange experiment has been done in a liquid phase containing a small amount of calcium, the liquid phase contains peptized material with a specific activity higher than the solid but lower than the liquid. Thus, the exchange percentage that we obtained was the ratio between the solid phase ($\times$ 100) and the liquid phase containing a fraction of the solid. This peptized phase, which may exist in a liquid phase that looks absolutely clear, may be partly eliminated by centrifugation at 15,000 rpm.[45] The amount of peptized material increases when the pH of the liquid phase is high. This is the case when alcohol-washed mineralized bone is used (Gabriel's method) that contains K_2CO_3 insoluble in this alcohol. Moreover, K_2CO_3 reacts with the tagged calcium of the liquid phase as soon as the solid phase is dispersed. The resultant $CaCO_3$ precipitate mixes with the solid and, by blocking the Ca^{45++} ions, disturbs the exchange equilibrium completely. It must be stressed that when bone mineral has been heated previously to 400° C., exchange values of 60 per cent or more can be reached. It has been discussed elsewhere[51] how the peptization of a part of the solid phase may determine in appearance such high exchange percentage values; the occurrence of pyrophosphate groups in bone heated to 400° C. (see footnote, p. 135) is probably very important in explaining why these surprising exchange values reach a maximum at this temperature. At the present time experiments are performed in order to discover the mechanism involved in this process. It is also interesting to note that the composition of the peptized phase depends on the calcium contents of the liquid

phase. The Ca/P weight ratio of the colloidal phosphate varies between 1.86 (peptization obtained without calcium) and 5.0 (with calcium).[48]

However, the various problems that we encountered can be avoided. K_2CO_3 may be eliminated from alcohol-washed mineral with formamide. When water-washed bone salts are concerned, the liquid phase may be cleared of any peptized phase by centrifugation at 15,000 rpm for a sufficient time; i.e., at least 4 hours. Finally, peptization itself may be avoided if exchange occurs in a *liquid phase with a pH near 7.0 and if* the bone particles used for the exchange experiments are not too small.[48,51] Movements of calcium between the solid and the liquid phase are abolished if the labeled calcium concentration of this liquid phase is adapted exactly to the amount of the solid phase.

3. RECRYSTALLIZATION OF BONE MINERAL

Logically, as Neuman and co-workers believed,[45] when bone mineral is suspended in a tagged calcium or phosphorus solution, surface exchange occurs rapidly. But at the same time a much slower phenomenon starts with the secondary movement of isotope into the crystals. This is in accordance with the old concept of Hevesy,[27] that some crystals dissolve in water, and simultaneously other crystals grow by apposition of new layers at the expense of the dissolved material. In this way, more and more isotope ions are trapped slowly in the bulk of the solid.

The views of Kolthoff[32-34] about recrystallization are quite different. For this author, recrystallization and ageing of crystals depend only partially on the same mechanism. Parallelly, crystal masses grow by agglomeration of small particles, and ions move from the surface into the bulk of the crystals by thermal vibration Initially Neuman did not agree with this concept because the diffusion of polyatomic groups, such as PO_4 groups, seemed to be difficult in such a compact lattice as calcium phosphate. Instead, he believed that recrystallization could occur only in a water phase and was temperature *dependent. But, later, concepts changed,* and Neuman and Weikel[46] discussed the thermal ejection of ions in the hydration layer of the crystal surface as well as the thermal vibration of ions into the bulk of the crystal.

We performed some experiments in order to obtain information about recrystallization. It was stated previously that if calcium-*exchanged bone mineral were leached with* dilute HCl, the isotope would appear to be located inside the crystal and not at the surface. Later we repeated the same experiments on bone salts exchanged during a shorter time and, by using this single modification in conditions, attained different results.[11] The greatest amount of isotope was located at the crystal surface. In other words, we found the largest concentration of isotope in the first leaching acid solutions. The deepest location of the isotope in the crystals correlated with the longest exchange time. Also, with time, the distribution of the isotope became rather homogenous in the bulk of the crystals.

It may be concluded that Kolthoff's views were confirmed, but it was not possible to assume that the phenomenon depended either on an aggregation of the crystals or on a migration of the surface-exchanged ions into the crystal. However, the x-ray diffraction data did not favor the first mechanism because no crystal growth was demonstrated. We think that this latter point is not conclusive, because aggregation of the crystals can be nonoriented, consequently not demonstrable by the x-ray diffraction lines broadening method. Another argument for the second mechanism was that the dispersion of the isotopes in the crystals was very homogenous. Moreover, we observed that the migration of the surface-exchanged ions occurred in the dry state. Bone mineral exchanged, dried and kept at room tempera-

ture during 3 weeks showed the same migration of surface ions. This migration is closely temperature dependent, the isotope being distributed homogeneously after heating at 400° C. for 24 hours. In this way we have obtained at least indirect evidence that thermal vibration is the main mechanism that may be evoked.

4. PERCENTAGE EXCHANGE VALUE

When exchange experiments with calcium are performed under conditions that circumvent the disturbing factors discussed above, the exchange percentage value for bone salts is approximately 10 to 12 per cent when determined in aqueous medium.[10,21] It is so also for dentin salts and for synthetic phosphates. The same experiments have been repeated with phosphorus, and the exchange percentage was the same.[3]

The third principal component of bone and dental tissues, namely, CO_2, has also been studied by means of $NaH^{14}CO_3$. Parallelly, the calcium exchange reaction was measured on bone mineral, total bone, enamel (with different particle sizes), dahllite and synthetic calcium phosphates containing a small amount of CO_2. The conclusions reached as a result of these experiments were as follows:

Only a fraction of the CO_2 content of the different compounds that have been studied is exchangeable. Thus, the greatest part of the CO_2 is located inside the crystals, provided that the particles are very small (less than 1 μ). With enamel, it has been established that the rate of the exchange is closely dependent on the particle size. The exchange percentage of CO_2, Ca and P in all cases is approximately the same; however, under the same conditions the exchange percentage of CO_2 (18%) is slightly higher than for calcium (11%).[22] It must be stressed that these experiments were performed on material that was stirred at least 24 hours in a nonlabeled $NaHCO_3$ solution, the high specific activity $NaH^{14}CO_3$ being added afterward. Such a method was used to avoid transfer of ions between the solid and the liquid phases in the presence of the labeled CO_2.

All these exchange experiments, with Ca^{45}, P^{32} or C^{14}, were performed first on a material definitely altered by mineralization and second in aqueous medium, which means that, paralleling exchange, remodeling phenomena occur (of unknown importance). In order to avoid any remodeling before and during exchange, we performed many new experiments comparing exchange percentage values of total and mineralized bone (without any contact with water) determined in alcoholic $Ca^{45}Cl_2$ solutions. The results confirm that early remodeling phenomena play a great part in the uptake of radiocalcium from an aqueous $Ca^{45}Cl_2$ solution, even if an apparent chemical equilibrium between the solid and the liquid phases was realized previously.[49,50]

CONCLUSIONS

Much work has been done since isotope studies with bone and teeth mineral commenced about 20 years ago. Since that time, labeled phosphorus, calcium and carbon have been used for different purposes. Successively, isotopes have been employed in facilitating chemical studies (1) to determine the surface properties of biologic phosphate crystals; (2) to obtain information about their molecular structure; and (3) to follow the remodeling of crystals or the interior migration of the surface ions.

The principal purpose of our studies was to define bone and teeth salts. We believe that the main conclusion to be drawn from these studies is as follows:

Bone and dentin mineral isolated from the organic matrix and studied by means of isotope method behaves as do hydrated synthetic phosphates. Synthetically, compounds closely proximating bone, dentin or cementum may be precipitated under well-defined conditions. However, the chemical composition of the biologic and the synthetic compounds is not absolutely the same, since

these phosphates do not rise in a liquid phase with the same composition as fluids circulating in bone. Thus, when we try to discuss the comparative molecular structure of the synthesized compounds, we must keep the following question in mind: Does this material behave in a manner similar to that of the mineral present in bone and teeth? Probably not.

Some arguments have been presented that lead to the belief that when the organic matrix of biologic hard tissues has been removed, the distribution of isotopes is altered. Most likely the principal role played by the organic fraction of bone and teeth is the stabilization of some kind of mineral structure that has not yet been clearly established. Any treatment used to obtain a pure mineral phase induces remodeling that disturbs the physicochemical properties of this material. It must be stressed that bone and teeth should be considered as a whole, each fraction—mineral and organic—influencing greatly the properties of the other. In this way, experiments dealing with exchange in nonaqueous medium may throw new light.

However, the work performed on biologic phosphates has been fruitful. The facts observed by us never contradicted the concept of the logical role played by the skeleton in the body; i e., as a storage depot of calcium, phosphorus and carbon dioxide. The crystals, even greatly remodeled, must be regarded as a building of ions—ions that are able to migrate into, as well as out of, the structure.

Probably because of this remodeling, the apparent exchangeability of Ca, P and CO_2 measured in aqueous medium seems to be the same. We must exclude the conception of an accumulation of PO_4^{---} or CO_3^{--} groups on the surface of crystals—at least when the isolated mineral fraction is concerned. Up to now it has been difficult to establish a comparison between the crystallization occurring in vitro and metabolic remodeling already observed in vivo The 8 to 10 per cent exchange that we obtained in alcoholic medium is the same for bone mineral and synthetic phosphates. This exchange depends somewhat on a surface area, which is not necessarily related to the apparent size of the ground particles of mineral. Accessibility of this surface area also is salient when bone, dentin and cementum minerals are concerned. Mineralized bone is a porous material, hence presents a considerable accessible surface area, contrary to what is observed for total bone powders and slices. Enamel is a very compact structure, and the surface of the elementary crystalline units is not accessible for exchange. The apparent volume of these particles, which may be measured with a microscope and a scale, is related directly to the exchangeable surface. (This surface area may be increased by grinding.) Removal of the organic fraction of enamel does not increase the porosity of this material enough to allow penetration of the isotope solutions. For this reason isotope studies of enamel yield poor results concerning crystal structure.

It seems that results obtained by in vitro radioisotope studies on biologic mineral materials should not be correlated directly to fundamental questions raised by the physiology of living organisms. Instead, they answer problems related to a pure chemical or physicochemical point of view, being especially helpful in the study of crystal agglomeration, ionic migration by thermal vibration, growing and remodeling of crystals.

Even now, the real structure of the crystals as they are present in the skeleton is not sufficiently defined. However, the determination of the structure of tricalcium phosphate by the infrared spectrographic method will help us to understand many facts that have already been observed and frequently misinterpreted. Moreover, the discovery of pyrophosphate groups in bone heated below 600° C., indicating the presence of hydrated tricalcium phosphate in this material, is very promising and could clarify the relations between the organic and the inorganic fraction of bone and teeth.

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Le Studio del Sales del Ossos per Medio de Isotopos

Summario in Interlingua

Es revistate previe utilisationes de isotopos in le studio del minerales de osso. Attention special es prestate al objectivos de ille labores.

Per exemplo, radiophosphoro ha essite usate a bon successo in studiar le adsorption de ille elemento (que es detegibile in micrissime amontas) per le crystallos presente in osso e in establir le composition de superficie de ille crystallos. Le conception del fixation de isotopos per le minerales ossee esseva alterate quando accurate determinationes chimic esseva effectuate in conjunction con le determinationes isotopic, e le principio de

un genuin reaction de excambio esseva definitemente establite.

Tamen, iste reaction de excambio que occurre al superficie de microcrystallos es disturbate per varie phenomenos secundari, como per exemplo per le processos de recrystallisation e de peptisation. Il es le recrystallisation que es responsabile si le isotopo, jam excambiate al superficie, penetra in le massa del crystallo. Iste phenomeno depende probabilemente de vibration thermal. Illo occurre mesmo quando minerales de osso es immagasinate in stato sic. Peptisation corresponde al decomposition del micre particulas de solido que forma un suspension colloide con alte grados de activitate specific. Le phase de peptisation debe esser differentiate ab le phase liquide pro determinar le procentage de excambio. Studios autoradiographic de sectiones de osso total ha monstrate que le deposition de isotopo non es homogenee, sed illo deveni homogenee quando le componente organic es eliminate. Iste observation supporta le conclusion que quando le mineral del osso es separate ab le componente organic, il occurre un refractionamento in le presentia de aqua, e le proprietates del crystallos del osso differe ab lo que illos esseva previemente. Post iste refactionamento, le minerales de osso se comporta exactemente como hydratate phosphatos synthetic que ha le mesme composition.

Le procentages de excambio pro Ca, P, e CO_2 (studiate per medio de Ca^{45}, P^{32} e $C^{14}O_2$) pare esser plus o minus identic, al minus quando le mineral de osso ha essite refactionate in le presentia de aqua.

14

The Deposition and the Removal of Radium in Bone by a Long-Term Exchange Process*

R. E. ROWLAND†

The behavior of the alkaline-earth elements in bone is a study that has received great impetus in the postwar era. Because of this, a considerable body of information has been gathered that describes the behavior of calcium, strontium and radium in the bones of various laboratory animals, but, of necessity, our corresponding knowledge as to their behavior in human bone has lagged. Most of the work in this area has been concerned with the toxicity of the radioactive elements of this group, particularly Sr^{90} and Ra^{226}, and has been based on the philosophy that information on the relative toxicity of this whole group, obtained in animals, could be transferred to the human being if the toxicity of one of the elements in the group were known in man.

This philosophy is based on the fact that considerable toxicologic experience in man does exist for one of these isotopes, namely, Ra^{226}. While, of necessity, most of the attention and interest in human beings burdened with radium for many years must be focused on the problem of toxicity, it is clear that from a thorough study of these individuals knowledge of a more basic nature also can be developed. It is the purpose here to illustrate the type of information that can be gathered on the uptake and the removal of radium by a process that we call long-term exchange. In this exchange process, radium will be shown to be acting in many respects as a tracer for calcium; hence it will provide information regarding the metabolism of calcium in bone.

Many years after intake, radium in human bone was found to be distributed in local intense deposits, which have been termed *hot spots*. This type of distribution is a result of the typical exposure to this element. An individual who acquired radium internally, either through exposure while painting radium dial watches or while receiving radium therapeutically, was usually not exposed for more than a few years. Thus, only new mineral formation that took place during this period would be intensely labeled with radium. Such locations—the hot spots—are often secondary haversian systems, such as shown in Figure 1. However, it has not been widely recognized that a remarkably uniform and widespread distribution of radium throughout all the bone accompanies this spotty distribution. This distribution of activity, called *diffuse* from its appearance on the autoradiograph, is easily overlooked, yet perhaps is more significant metabolically than the well-known hot spots. The gross

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autoradiographs shown in Figures 2, 3 and 4 are presented to illustrate this diffuse distribution. In general, the diffuse distribution is quite uniform and seems to be present in all the bone. Figure 4, however, has been included to show that some variations in the diffuse specific activity can, and do, occur.

In a recent study[1] of the distribution of radium in human bone, quantitative evaluation of the specific activities found in the hot spots and in the diffuse distribution has been reported. This study included 12 individuals who had carried this isotope for at least 20 years. The specific activities, ex-

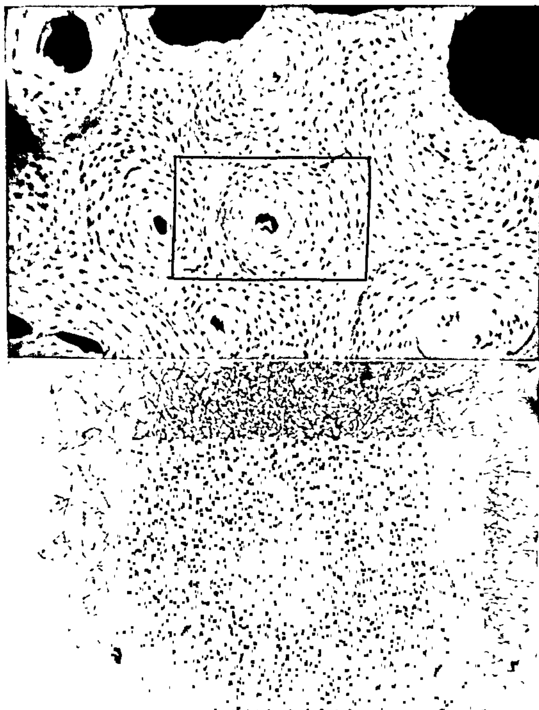


FIG. 1. (*Top*) A microradiograph (× 142) and (*Bottom*) an alpha-track autoradiograph (× 433) of a bone section from the femur of a woman who acquired radium therapeutically at 36 years of age and died 28 years later with a burden of 6.8 μc. The stripping film autoradiograph covers the outlined area of the microradiograph.

of one calcium atom into one bone crystal is balanced instead by the loss of a single atom from another crystal. Whatever the process, a statistical balance is achieved over the microscopic mineral volume.

These Ca^{45} studies in dogs have demonstrated the existence of a mechanism that results in a diffuse distribution of this isotope throughout the bone. Since Ca^{45} is employed as a tracer for stable calcium, it is evident that a transfer of the latter from blood to existing bone mineral is taking place. The transfer rate from blood to bone, termed the "augmentation rate,"[1] is evaluated *by dividing the measured specific activity* (a) of a diffusely labeled volume of bone at a time (T_s) after an intravenous injection of the isotope by the time integral of the specific activity of the blood (B). Thus, the augmentation rate (f) is given by:

$$f = \frac{a}{\int_0^{T_s} B \, dt}$$

where the integral extends from the time of injection to the time of sacrifice. This augmentation rate has been found to be age dependent, decreasing from a value of 0.2 Gm Ca/Gm. Ca-year in 1-year-old dogs to 0.05 Gm. Ca/Gm. Ca-year in a 12-year-old dog.

Having demonstrated this calcium transfer and measured its rate, we need to know whether or not the radium atom can substitute for calcium in this exchange process. As a test, the augmentation rate was measured in 2 dogs, a year or so of age, using Ra^{226} as the tracer. One month after injection the diffuse distributions were measured, and values of 0.26 and 0 20 Gm. Ca/Gm. Ca-year were obtained for the augmentation rates. These were in excellent agreement with the values obtained previously under comparable conditions with Ca^{45} and seem to indicate that this isotope, Ra^{226}, also takes *part in the exchange process at a rate similar* to that indicated by Ca^{45}.

It is somewhat surprising to find tl radium atom will act as a quantitative for a calcium process. On the basis alone, it is difficult to understand hc large radium atom can substitute for c; in the hydroxyapatite crystal. It is pr(a valid tracer only at extremely low c(trations. For example, the diffuse r distribution existing in an individual body burden of 1 μc. of Ra^{226} (10 tim accepted industrial maximum perm level) contains only 1 radium atom pe calcium atoms.

It should also be pointed out that the *vation that equal augmentation rates* for both Ca^{45} and Ra^{226} does not impl equally intense diffuse labeling would l *tained for these isotopes in a double-* experiment. In the dog, the plasr cleared of Ra^{226} more rapidly than it Ca^{45}, so that the time integral of the specific activity is less for radium tha calcium. Thus, even with equal augm tion rates, the radium diffuse level wi lower than the calcium diffuse level.

When we direct our attention to this term exchange process in the human b we have potentially a much longer peri observation than is possible in labor animals. In addition, since it appears Ra^{226} can be considered to be a trace calcium in these processes, we have an tope of much longer half-life than an the available calcium isotopes. An tremely unusual case illustrates clearly these processes go on continually in hu bone. A 51-year-old male terminal with an osteogenic sarcoma, was give tracer dose of Ca^{45} intravenously. Tw or more years before he had received R probably as an intravenous medication, had retained 1.2 μc of Ra^{226}. An amp tion had been performed some time be the Ca^{45} tracer was administered, so radium-labeled bone was available for an sis At autopsy, 23 days after Ca^{45} adr istration, a second bone sample was *tained that was labeled with both* Ca^{45} Ra^{226}.

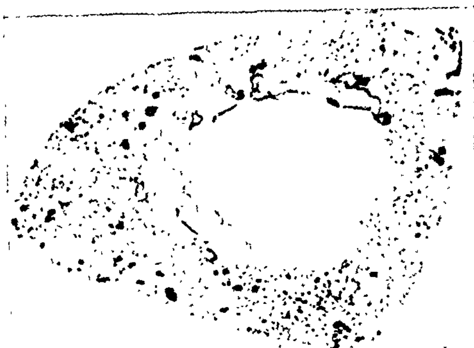


FIG. 5. An autoradiograph of complete tibia cross section (× 4.7) from a 41-year-old man who had received Ca^{45} intravenously 23 days prior to death. Note the remarkably complete labeling of all the compact bone with the tracer.

In Figure 5 the autoradiograph of this Ca^{45}-labeled bone is shown.* The illustrated Ca^{45} deposition indicates that this compact bone is still exchanging calcium atoms with the blood. The augmentation rate, calculated from the Ca^{45} label and the 23-day integral of the plasma specific activity, is 0.02 Gm. Ca/Gm. Ca-year, a value somewhat less than the corresponding value in adult canine bone. This should not be interpreted to mean that, after the intravenous injection, the uptake of these isotopes is lower in man than in the dog, because it must be remembered that the total uptake of the tracer by the exchange process is given by the product of the augmentation rate and the time integral of the blood specific activity. In human beings, the plasma is cleared of Ca^{45} at a lower rate than in the dog; hence, in humans, the exchange process has a longer time over which to label the compact bone.

If our assumption that the diffuse distribution of these isotopes in compact bone represents a deposition by a long-term exchange process is correct, then we would expect that the magnitude of the diffuse distribution of a tracer not supported by continual uptake would decrease with residence time in bone. That is, the label is not fixed permanently within the mineral but eventually will be removed by the same exchange process. Two types of evidence are available that support the conclusion that the magnitude of the diffuse label decreases with time.

Marshall[1] has reported measurements of the diffuse distribution in dogs that indicate that a loss of activity does occur. Using Ca^{45}, the skeleton of a 1-year-old beagle was examined at two different times: first, a month after injection of the isotope by means of an amputation of the radius-ulna; second, a year later when the opposite leg was obtained at sacrifice. The diffuse specific activity was found to have decreased by 10 to 20 per cent during this time in regions of compact bone not subject to resorption. He also demonstrated a similar loss of diffuse activity, employing radium as the label, in a group of 11 beagles analyzed autoradiographically at different times after injection. It was found that animals sacrificed 4 years after injection showed approximately one half of the diffuse specific activity of animals sacrificed a few months after injection.

A less direct, but probably equally signifi-

* A section of bone from the previously amputated limb, containing only Ra^{226}, was exposed on this plate for the same time (21 days), but no diffuse darkening from the radium was visible. However, when the radium section was left for 6 months on a similar plate, the radium diffuse distribution also could be seen

cant, argument for the continual loss of activity from the diffuse distribution is implied in the general observation that the diffuse specific activity is about one half of the hypothetic uniform label, irrespective of time after injection. This has been found to be true of all the dogs studied at various sacrifice times in this laboratory, employing either Ca^{45} or Ra^{226}, as well as of the 12 human radium cases mentioned previously. Since it is well known that the total skeletal content of these elements in both man and dog decreases continually with time after injection, it follows that the magnitude of their theoretic uniform label, hence the diffuse specific activity, also must decrease with time.

Thus, evidence has been assembled showing that a calcium-exchange process is responsible for the diffuse distribution of radium in human bone. This radium is not fixed irreversibly within the compact bone mineral; rather it is removed in time by the same exchange process that deposited it originally. The rate at which this activity is removed is dependent on the fraction of the total number of calcium atoms in bone that are available to take part in the exchange process. For example, if only one fifth of the calcium atoms were available for exchange within a given microscopic volume of bone, then the diffuse distribution acquired by exchange would be removed 5 times more rapidly than if all the calcium took part in this process. Augmentation rate measurements, which give the transfer rate of calcium ions from blood to bone, give no indication of the size of the calcium compartment involved. However, when these measurements are coupled with studies on the rate of removal of a diffuse distribution, the magnitude of this compartment can be defined. The preliminary results from the dog studies, as well as the observations of human bone, seem to indicate that a sizable fraction, if not all, of the bone calcium is available for this exchange process.

It should also be pointed out that, following short-term administration of a tracer isotope, at least two other mechanisms of calcium deposition in bone can produce a long-lived distribution of intensity similar to that produced by the above-mentioned exchange process, but more limited in area. The first of these is new mineral formation. In this instance, new mineral apparently is formed at the specific activity of the blood plasma, so that at a short time after, say, radium is present in the plasma, the mineral formed will contain a relatively high radium specific activity. In this manner the hot spots are produced. As the plasma radium level drops, subsequent new mineral is formed at lower specific activities, and eventually, after a year or so, the new mineral contains radium levels lower than the original diffuse distribution. A second mechanism of deposition of calcium in bone, hence of a tracer, is termed "secondary mineralization." New bone mineral, particularly in secondary haversian systems, is formed at a relatively low mineral density. Subsequently these systems increase slowly in mineral density and so are able to acquire a tracer by a true accretion process. Each of these processes can produce tracer distributions of specific activities that are indistinguishable from the over-all diffuse distribution, but even together they cannot produce the widespread diffuse distribution that always results after administration of an alkaline-earth isotope.

It will be of interest to ascertain whether or not the loss of a tracer from a hot spot deposit, i e., new mineral formation, occurs at the same rate as the loss from the diffuse distribution, i e., exchange deposition. Studies of this nature could also be carried out over long periods of time on human bone with radium and should extend our knowledge of the metabolism of calcium in bone. It may even be possible to compare the rate of loss of the radium tracer acquired by the process of secondary mineralization with the rate of loss observed in hot spots and diffuse distributions to complete the over-all picture.

The exchange process delineated by the diffuse distribution of radium in human bone is not of much metabolic significance in the

minute-to-minute regulation of the blood calcium levels, for apparently it only transfers some 50 mg. of calcium per day in the adult human. However, this process achieves its importance now, in the era of radioactive fallout, since it accounts for a sizable fraction of the retained burden of calcium and radium tracers and, therefore, undoubtedly does the same for Sr^{90}.

Thus it becomes of interest to speculate on the regulation of this process: is it regulated biologically, or is it simply the manifestation of a physical exchange and diffusion process? Apparently the rate decreases with bone age. It remains to be seen whether the decrease is the consequence of a growth in the average size of bone crystals, such as postulated by Neuman and Neuman,[3] resulting in a longer residence time for the tracer atoms, or is due to other mechanisms. If the process is regulated biologically, the possibility exists of increasing the rate at which it operates in the hope of removing unwanted contaminants from bone mineral.

In summary, the diffuse distribution of radium remaining in the mineral of human bone some 20 to 30 years after exposure to this isotope has been analyzed quantitatively and found to be a significant fraction of the body burden. Coupled with the uptake studies of Ca^{45} in dogs, it is postulated that this distribution is the result of a general calcium exchange from blood to bone, characterized by a long-time constant. The fact that the distribution persists in human bone for so long a period is not an indication that the radium is "trapped" in inaccessible bone mineral but, rather, that the turnover rate for calcium in mature bone mineral is low. Concurring evidence is accumulating that this isotope is removed slowly from the mineral, without direct resorption taking place, apparently by this same exchange process that deposited it originally.

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Le Deposition e Elimination de Radium in Osso per Un Processo de Excambio a Longe Vista

Summario in Interlingua

Studios quantitative del distribution de radium in osso human provide un methodo pro obtener informationes relative al metabolismo de calcium in osso le quales non es obtenibile per technicas conventional. Le diffuse distribution de iste isotopo radioactive es considerate como un exemplo; es postulate que iste distribution es le resultato de un processo de excambio a longe vista que continuemente transfere calcium ab le sanguine ad le osso e, in le curso del tempore, de novo a retorno. Es monstrate que radium es un traciator de calcium in iste processo, al minus a bassissime nivellos de concentration, como illos existe in humanos exponite a radium—therapeuticamente o occupationalmente—trenta o plus annos retro. Viste le basse observate nivello de intensitate con que iste activitate es eliminate, le these es presentate que pauco o nihil del calcium de osso es de facto exempte ab le mentionate processo de excambio.

15

The Radioisotope Osteogram—Kinetic Studies of Skeletal Disorders in Humans*

NORMAN S. MACDONALD

During the last 15 years there has been a dramatic increase in the intensity of experimental research devoted to bone as a tissue. In the United States a number of research programs encompassing broad studies of bone physiology have been developed and supported by the Atomic Energy Commission.

One important factor in the heightened interest in bone metabolism is, of course, the quite justifiable concern over the effects of the increased amounts of radioactive materials now entering the food supplies of human populations. Certain of these radioisotopes, notably Sr^{90}, are readily absorbed and deposited in the bony tissues. There is still another factor behind this rapid growth of interest in bone physiology—the influence of Franklin C. McLean. His many important contributions to the scientific literature are a matter of record, but, in addition to this, his untiring behind-the-scenes efforts to initiate and support symposia, conferences and lectures dealing with bone physiology have been of great value in stimulating the advances in this field.

Out of the huge volume of experimental observations dealing with the biologic behavior of potentially hazardous radioactive "bone seekers," such as radium and Sr^{90}, a number of fairly well-established principles have emerged. By suitable application of these it is feasible to use radioactive elements as "tags" or "tracers" for investigating the normal constituents of tissues.

The recent development of scintillation counters for gamma rays has made it possible to measure changes in radioactivity within the body by using collimated detectors held near the skin and pointed at various parts of interest. Data of this sort give information that often is unobtainable from assays of daily excreta. Unfortunately, the low penetrating power of the beta rays of Ca^{45} makes it unsuitable for measurement by detectors placed outside the body. Furthermore, the beta-gamma emitting isotope Ca^{47} is exceedingly expensive and difficult to

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procure. However, radiostrontium can be used as a tag for the study of bone calcium. Admittedly, strontium is not a perfect atom for this purpose; qualitatively, its absorption, transport, deposition and excretory patterns are quite similar to those of calcium, but quantitative differences do exist and must be taken into account.[1,6,11,15] The value of this technic of "body surface counting" of injected Sr^{85} and Ca^{47} has been clearly demonstrated by Bauer and others in several clinical studies of skeletal disorders.[3,4] However, relatively little emphasis has been laid on studying and *utilizing* the events that occur immediately following the entry of the bone-seeking radioactivity into the blood. Laboratory experiments with animals have indicated that important information regarding osseous tissue can be secured by recording, more or less continuously, the variations of radioactivity in body areas during the *first* hour after injection of bone-seeking radioisotopes and supplementary tracers, such as radiosodium and radioiodine. These graphic records were called radioisotope osteograms.[10] It is the purpose here to present an account of our first attempt to apply the osteogram technic to human subjects.

MATERIALS AND METHODS

Carrier-free Sr^{85} was purchased from the Nuclear Science and Engineering Corporation; radioiodinated serum albumin (RISA), from Abbott Laboratories. Sterile saline solutions of these materials were prepared Doses ranged from 5 to 15 μc. and were administered by direct injection into an antecubital vein.

The basic equipment consisted of a lead-shielded, collimated gamma scintillation probe with a 1½ inch × 1 inch sodium iodide crystal, photomultiplier tube and preamplifier whose output was fed to a scaler. The detector was mounted on a counterbalanced adjustable stand that could be taken to the patient's bedside. The usual procedure was to place an indelible ink mark on the surface of the skin over the area of interest. Several such spots were chosen; for example, temple, one or more vertebral bodies, knee or midshaft of a tibia, or a site of obvious skeletal pathology. Background counts were obtained with the detector touching the skin at each of the marked positions, after which the injection was delivered into a vein. A count of 1-minute duration was taken immediately at one of the chosen sites and recorded; the scaler then was reset, and the detector was aligned over the next site. This operation required less than 1 minute. A 1-minute count was taken and recorded, and the detector was moved to the next site. After all sites had been counted in this manner, the whole sequence was repeated several times. Thus, the raw data for the osteogram consisted of observed activity (counts per minute) at each area, as a function of time after injection. In a few cases these observations were continued for as long as 2 hours. When a continuous record was desired, with the detector fixed in one position, the scaler was replaced by a counting ratemeter and a strip-chart recorder. When it was possible, the patient was returned to approximately the same reclining position at intervals of 24 hours for postosteogram monitoring of the marked areas. Unfortunately, this could seldom be arranged, since most subjects either were outpatients or had left the hospital soon after the test.

Three subjects were in the Metabolic Ward (Medical Center, UCLA), so that balance data on calcium, phosphorus and nitrogen were available. For these patients it was possible to obtain consecutive values for the total 24-hour urinary and stool output of the administered Sr^{85}, as well as for a few serum samples All such samples were assayed for radioactivity by counting suitable aliquots in a stationary scintillation counter equipped with a single-channel pulse height analyzer. Activities were expressed as percentage of administered dose by comparing them with the counts observed the same day

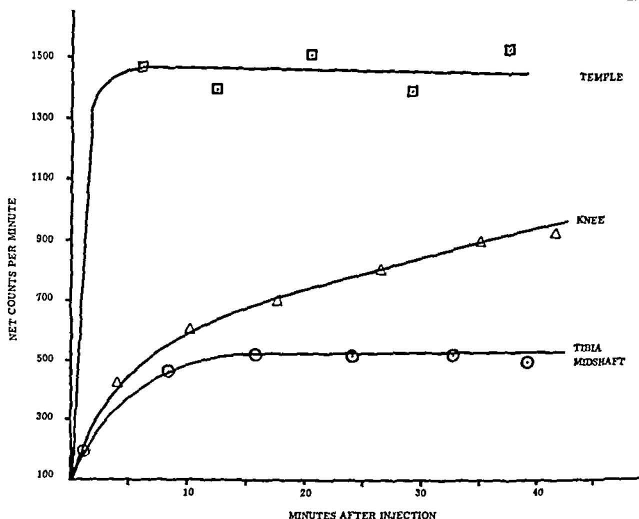


FIG. 1. Sr^{85} osteogram of 24-year-old female with no skeletal abnormalities.

in aliquots of the dose stock solution prepared so as to present the same geometry to the detector.

The earlier work with rabbits bearing tibial fractures[10] had shown the usefulness of observing the behavior of radioiodinated serum albumin (RISA) in conjunction with the Sr^{85} osteogram. When the present clinical studies were undertaken, adequate dual-channel gamma spectrometer equipment was not available, so that in the several dual-tracer studies performed it was necessary to inject the RISA first. The activity of the protein-bound I^{131} at the various sites then was measured at repeated 1-minute intervals as described above After the counting rates had become constant (usually within 10 minutes), Sr^{85} was injected, and the replicate counting procedure was carried out again. The Sr^{85} activity for each individual measurement was obtained by subtracting the counts per minute due to RISA from the observed total counts per minute. In future work a 2-channel gamma spectrometer will make it possible to count Sr^{85} and I^{131} simultaneously after injection of a mixture of two radioisotopes.

RESULTS

Figure 1 gives the osteogram for tibia midshaft, temple and knee obtained from an injection of 5 μc. of Sr^{85} administered to a "normal" 24-year-old white female with no known disturbance of skeletal structure or metabolism. In describing such curves it is convenient to refer to the time at which maximum radioactivity was observed. In Figure 1, for example, T_{max} was about 15 minutes for the tibia-midshaft position of

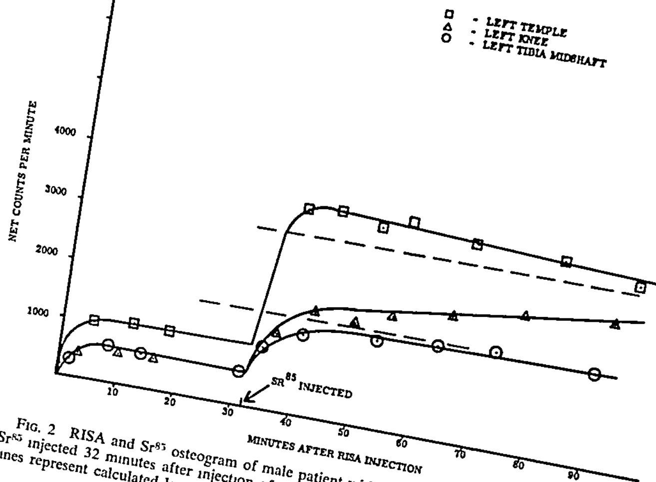


FIG. 2 RISA and Sr^{85} osteogram of male patient with multiple myeloma. Subject M-2. Sr^{85} injected 32 minutes after injection of radioiodinated serum albumin (RISA). Broken lines represent calculated level of activity if Sr^{85} had behaved as would RISA.

the detector, 6 minutes for temporal area and more than 41 minutes when the spongiosa of the knee was in the "field of view" of the broadly collimated detector. It is useful also to make rough comparisons between the various activities measured. Thus, the Sr^{85} in the blood, the soft tissue and the bone in the field of view of the detector, when directed at the temple, gave about 3 times the maximum counting rate observed when the detector was placed over the tibia-midshaft area. Interpretation of these relationships will be discussed later.

Figure 2 illustrates the RISA osteograms and the Sr^{85} osteograms of a male patient suffering from multiple myeloma. By counting separately the syringes containing the RISA and the Sr^{85}, when inserted into a plastic phantom, before and again after the injections, a rough approximation of the relative counting rates of I^{131} and Sr^{85} actually given the patient can be made. This value is used to estimate the levels of gross counts per minute (I^{131} plus Sr^{85}) that would be expected if the Sr^{85} were to behave exactly as RISA does in the body. The dotted lines represent these calculated anticipated levels. An index of the difference between the behavior of Sr^{85} and RISA can be obtained by computing the ratio

$$\left(\frac{\text{c/m of RISA}}{\text{c/m of } Sr^{85}}\right) \text{at body site} \div \left(\frac{\text{c/m of RISA}}{\text{c/m of } Sr^{85}}\right) \text{in dose.}$$

When this index is greater than 1.0, more Sr^{85} has accumulated at the site under investigation than would have been predicted merely on the basis of tagged blood volume in that site.

Table 1 presents some values for the times required for attainment of maximum activity at various body sites in a number of subjects. For those cases in which RISA also was administered, the accumulation index, A, also is given.

Table 2 illustrates the magnitude of Sr^{85} activity measured by the detector when "aimed" at various body sites—relative to the activity of the mid-tibial area expressed as unity. This comparison was made at 30 to 45 minutes after the Sr^{85} injection. However, it should be emphasized that the shape or *trend* of the osteogram record is its most important feature. The *changes* of radioactivity with the passage of time are of greater significance than any single measurement taken at some arbitrary time after the injection. Furthermore, it is futile to attempt to express the radioactivity observed by the detector in terms of "per cent of dose" or of absolute activity (disintegrations per minute) because the factors of geometry and scattering are unknown—and, indeed, vary from one position over the body to another.

DISCUSSION

Interpretation of the Osteogram

The volume of tissue "seen" by the collimated detector comprises skin, blood, lymph, muscle and other soft tissue, bone and marrow. Therefore, changes in the total observed radioisotope activity with the passage of time reflect progressive changes of

TABLE 1. ACCUMULATION OF Sr^{85} IN BONE AREAS

Subject	Skeletal Involvement	Tibia Midshaft		Knee		Temple	
		T_{max}	A	T_{max}	A	T_{max}	A
H-4		20	0.8	>40	1.7	—	—
H-5		15	0.5	>30	0.7	13	0.9
H-7	None (normals)	35	—	>60	—	12	—
H-13		25	—	>53	—	9	—
H-18		15	—	>45	—	5	—
H-19		20	0.8	>40	1.7	5	1.0
	Paget's disease	10 rt.	1.3	>45	3.5	15	1.1
H-20		>45 lt	2.3				
H-10	Osteoporosis	>35	—	>35	—	—	—
H-12	age >70 yrs.	>35	—	>35	—	5	—
H-14		>60*	—	>60	—	>60*	—
H-15		>40	—	>40	—	22	—
H-16	age 30 yrs.	45	—	>60	—	15	—
H-17	Healing hip fracture with osteoporosis ?	15	1.6	—	—	5	1.9
	Multiple myeloma						
M-2	Osteogram #2	30	1.0	>60	1.8	12†	2.2
	Osteogram #3	>45	1.0	>60	1.1	5†	1.3
H-21	Post-hypocalcemia	32	1.6	>60	1.8	15	1.2

A = accumulation index = (Sr^{85}/RISA) in bone ÷ (Sr^{85}/RISA) in dose, at 1 hr. after RISA injection
T_{max} = minutes after Sr^{85} injection when maximum counts per minute was reached
* Attributed to faulty injection—some Sr^{85} subcutaneous at point of injection.
† Activity declined slowly after reaching maximum level

the amounts of radioisotope residing in each of these tissue categories. At these early times following the injection it is manifestly impossible to state with confidence what fraction of the Sr^{85} activity observed at any chosen moment is due to Sr^{85} in blood, what fraction is in soft tissues and what portion is deposited in bone. Some attempts to attack this problem by double-tracer experiments on rabbits, using Sr^{85} in conjunction with Cr^{51}-tagged red cells, diffusible ions such as Na^{22} and nondiffusible tagged serum albumin have been reported.[10] It was concluded that the speed of mixing and transport of the injected radioisotope within the vascular network was rapid enough to ensure that the maximum concentration of Sr^{85} within these capillaries in a bone area was reached well within 8 minutes. This has been borne out in the clinical studies reported here; when RISA (which presumably cannot diffuse rapidly through the capillary walls into extravascular spaces) was administered intravenously, the counting rate always reached a maximum in less than 10 minutes and usually in about 5 minutes, after which it remained constant. In many situations, however (see Table 1), maximum counting rates for Sr^{85} were reached only after many minutes or even many hours (for example, knee activity increased for 24 hours in most cases). Therefore, the behavior of the Sr^{85} counting rate after the first 8 to 10 minutes is probably determined by what happens to the radioactive ions after they have been mixed thoroughly with the blood and transported to the area under the detector. A large number of processes, of varying degrees of reversibility and quantitative importance, can be postulated as governing events in the fate of Sr^{85} ions during the first hour or so. Among these are diffusion or transport across the capillary membrane into extravascular fluids; transport into the interior of cortical bone via haversian canals and canaliculi; ionic exchange with an equal number of nonradioactive ions at the surfaces of bone mineral crystallites and possibly within crystal hydration layers;[13] sequestration within the bulk of newly form-

TABLE 2. MAXIMUM SR^{85} ACTIVITY OF BODY SITE RELATIVE TO RIGHT TIBIA MIDSHAFT AS 1.0 AT 30 TO 45 MINUTES AFTER SR^{85} INJECTION

Subject	Skeletal Involvement	Knee	Lumbar Spine	Temple
H-4	None (normals)	1.9	—	—
H-5		>1.1	4.1	1.7
H-7		1.6	4.0	2.8
H-13		1.7	4.9	2.1
H-18		1.8	7.6	3.0
H-19		2.3	—	2.9
H-20	Paget's disease right tibia = 1.0 left tibia = 2.5	1.4 (rt)	3.5	1.8
		2.3 (lt)	—	—
H-10	Osteoporosis age >70 yrs.	2.3	—	—
H-12		1.5	7.3	3.0
H-14		1.7	—	3.5
H-15		1.6	—	2.1
H-16	age 30 yrs.	1.4	4.9	2.0
H-17	Fracture, hip	1.4	8.3	2.6
M-2	Multiple myeloma	1.5	8.0	2.2
		1.2	—	1.6

ing bone mineral during accretion or apposition of new bone[7] and binding with components of soft tissue[15] and the organic matrix of bone.[10]

The results of this exploratory clinical study do not offer incontrovertible evidence elucidating the operation of any of these processes. However, the results do show that by radioisotope technics it is possible to demonstrate serious deviations from their normal operation.

Osteoporosis

The osteograms of 5 patients with a diagnosis of osteoporosis differed from normals by exhibiting a distinctly more sluggish accumulation of Sr^{85} at sites comprised primarily of cortical bone (Table 1). Thus, the detector aimed at the mid-tibia region registered a slowly increasing counting rate during the whole examination, which lasted at least 35 minutes. With the 6 normal subjects, however, this area reached a maximum activity usually in less than 25 minutes and remained level. Experiments are currently under way to determine whether or not impaired circulation in senile osteoporotic subjects plays a part in causing this effect.

Paget's Disease

One patient with this malady—a 46-year-old female—was examined. The middle third of the left tibia was tender, swollen, bowed and hyperemic, but without severe pain. The right tibia seemed to be unaffected. Figure 3 shows the RISA and the Sr^{85} osteograms. The left limb showed a higher level of RISA activity, reflecting the greater volume of blood coursing through this afflicted area. More significantly, Sr^{85} continued to accumulate in the affected segment all during the test, whereas the unaffected right tibia reached maximum activity in 10 minutes and remained at that level. What particular process in the welter of metabolic chains operating within this hyperactive area is

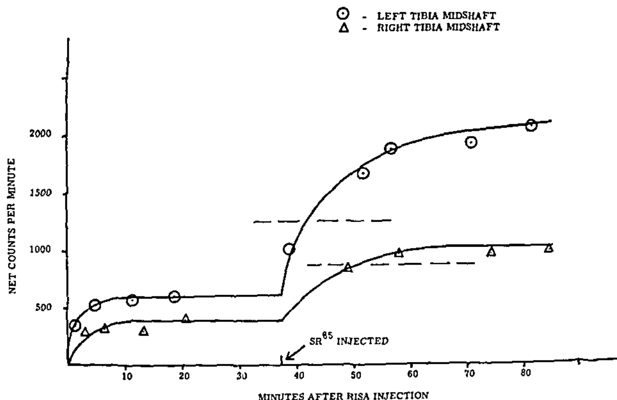


Fig. 3. RISA and Sr^{85} osteogram of a 46-year-old female with Paget's disease. Left tibia bowed and hyperemic. Sr^{85} injected 37 minutes after injection of radioiodinated serum albumin (RISA). Broken lines represent calculated net activity if Sr^{85} were to behave as would RISA.

responsible for this avid accumulation of the tracer strontium? The data offer no direct clue. One interesting possibility is that an extremely rapid turnover of calcium occurs in the lesion; that is, abnormally large quantities of bone salts constantly are being resorbed and replaced by newly formed mineral with little net gain or loss in bone density.

Multiple Myeloma

A rather complete study of Sr^{85} metabolism was carried out on one subject (F.H.) with a well-established diagnosis of multiple myeloma. Over a period of 6 months, 3 separate injections of Sr^{85} were administered. The excretion data will be discussed later. The osteograms for tibia and knee did not differ sufficiently from our meager data for normals to warrant attachment of any significance (see Fig. 2 and Tables 1 & 2). However, the Sr^{85} activity registered when the detector was aimed at the temporal area decreased over a period of 30 minutes, after attaining its maximum value at 5 to 12 minutes after injection. This early decrease was not observed in our series of normals. Roentgenograms of the patient's skull did show "punched-out" lesions, but with our very limited experience it is impossible to say whether or not the temple osteograms truly reflected this pathologic condition of the skull.

Metastases of Tumors

Bone-seeking radioisotopes can be used to gain information to supplement roentgenographic findings. An example of this was provided by a 42-year-old female with an

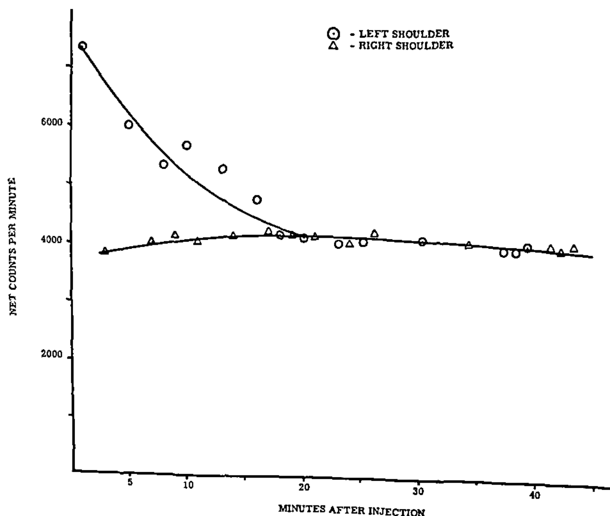


Fig. 4. Sr^{85} osteogram of a 42-year-old female with calcified lesion near head of right humerus.

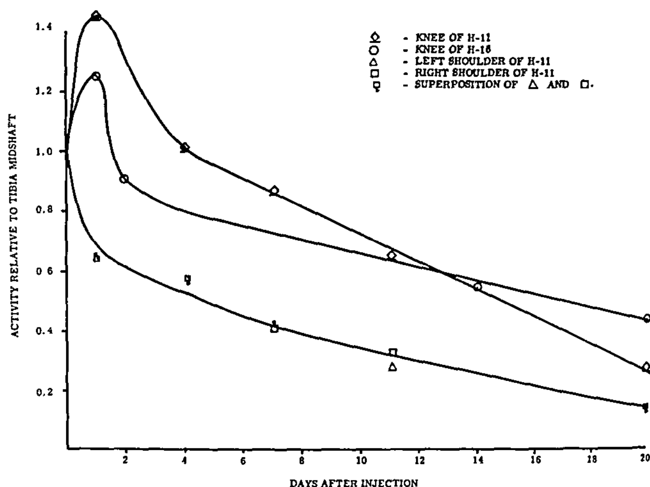


FIG. 5. Monitoring of areas after injection of Sr^{85}. H-11 had a calcified lesion near head of right humerus and metastases from a breast tumor H-16 had osteoporosis.

infiltrating carcinoma of the right breast (biopsy). The roentgenographic findings indicated osteoblastic lesions in the pelvis and the humerus. The latter lesion appeared as a large well-calcified body near the head of the right humerus. The left arm appeared to be normal. The patient lay supine, and Sr^{85} was injected into the left antecubital vein. Repeated counts were made with the detector placed alternately on each shoulder, directed vertically downward toward the head of the humerus. Figure 4 presents the results. Initially the activity in the left shoulder was almost twice that in the right shoulder area, but it fell rapidly and by 20 minutes matched it almost exactly. Why the activity in the left shoulder area followed this pattern during the first few minutes is not certain. It is possible that faulty injection may have deposited some of the isotope material into subcutaneous tissue in the left forearm. Relatively slow absorption into the blood and transport toward the heart would bring steadily decreasing amounts of this radioactivity under the detector (when it was located over the left shoulder) before it was mixed thoroughly throughout the blood. Regardless of whether or not this is the correct explanation, the important observation is that after 20 minutes the area encompassing the supposed osteoblastic lesion contained the same quantity of Sr^{85} as the normal contralateral area This equivalence was verified by 5 subsequent counts, the last of which was 20 days after injection (see Fig. 5). The most plausible conclusion is that the well-calcified mass seen in the roentgenogram was *not* in a state of active calcification at the time of examination. There was not any great turnover of calcium; nor was there

any significant apposition or accretion of new calcium salts within the mass. This interpretation was supported to some extent when a search of the patient's records uncovered 2 roentgenograms taken 6 months and 2 years previously. In all 3 plates the calcified mass appeared to have the same density and dimensions.

One final example may be cited to suggest the potential utility of the osteogram technic. An 85-year-old male, suffering from cancer of the prostate, was tested with RISA and Sr^{85}. The x-ray findings had indicated extensive osteoblastic metastases to bone, with bilateral involvement of the pelvis, somewhat more pronounced on the right side. Replicate counting over the left and the right ilium showed that tagged serum albumin reached maximum concentration in both sites in from 4 to 5 minutes and remained constant. However, when Sr^{85} was injected, activity in the left iliac area still was increasing at the end of 25 minutes, whereas activity on the right side reached a maximum in 14 minutes and remained constant. These radioisotope findings suggest that metabolic activity involving calcium turnover was much greater on the left side of the pelvis than on the right.

Monitoring Data

Although the measurement of radiostrontium activities during the first hour after injection can yield very illuminating information regarding the broad features of calcium metabolism, the full picture can be seen only when retention and excretion data also are at hand. Retention of gamma-emitting radioactive isotopes within the body as a whole can be measured in "total body counters," two prototypes of which were developed at Argonne National Laboratory and Los Alamos Scientific Laboratory. Comparisons of the retentions by various parts of the body can be made by monitoring with a movable detector equipped with adequate shielding and collimation. Unfortunately, our data to date on the day-by-day variation in radioisotope activity in the bones of the patients tested are too fragmentary to permit of useful generalizations linking such measurements with specific skeletal disturbances. Sr^{85} activity in tibia shaft and skull begins to decline a few hours after injection. Areas containing spongiosa or localized spots of intense metabolic activity (healing fractures) continue to accumulate activity for 24 hours or more before declining. (Similar findings already have been reported by Bauer, Carlsson and Lindquist,[4] who have amassed excretion and bone monitoring data on several hundred cases.) Figure 5 illustrates the sort of information obtained by monitoring measurements. One of the subjects (H-11) is the same patient discussed earlier who had a calcified mass of probable metastatic origin in the right shoulder. It is apparent that this pathologic mass has had little effect on the deposition and the retention of Sr^{85} in the area and, therefore is, relatively inactive in terms of calcium metabolism.

It should be noted that, with the exception of the knee area for the first day or two, all the areas monitored lost Sr^{85} at a faster rate than the tibia midshaft. That is, for any given day, the ratio of shoulder activity/tibia activity always was less in magnitude than the ratio at 1 hour after injection. This does not *prove* that these *bone* tissues lose Sr^{85} at a faster rate than tibia cortex (although this may very well be the case) since there is considerably more soft tissue and blood in these areas than in the tibia midshaft. Clearance of Sr^{85} from these soft tissues, being more rapid than from bone, may account for a goodly part of this rapid decline in total activity detected by the monitoring instrument.

Excretion Measurements

Although measurements of the daily output of a radioactive tracer in the urine and stool can provide very accurate information on the retention in the body, usually the cost in time, effort and money is prohibitive.

TABLE 3. URINARY EXCRETION OF RADIOSTRONTIUM

Isotope	*Patient*	*a*	*b*	*Condition; Treatment*
Sr^{85}	F.H. Test No. 1	28.6	−1.7	Multiple myeloma (prednisone)
Sr^{85}	F.H. Test No. 2	20.0	−1.5	(prednisone and testosterone)
Sr^{85}	F.H. Test No. 3	18.5	−1.4	(testosterone and ACTH)
Sr^{90}	50-year-old male		−1.1	Normal*
Sr^{90}	20-year-old female		−1.2	Normal*

$E = at^{-b}$, where E = % of dose excreted per day.
t = time, in days, after injection.
b = slope of the line when E and t are plotted on log-log co-ordinate paper.
a = intercept on the E axis; corresponds to the % excreted during Day 1.
* Reported by Stewart *et al.*[16]

However, one patient (F.H.) with multiple myeloma, who was maintained in the Metabolic Ward for other purposes, was available for 3 Sr^{85} studies during his course of treatment. In each case, excretion in urine and stool was followed for at least 30 days after administration of the radioisotope. One very useful way of handling such data is to plot, for each sample, the logarithm of the 24-hour output against the logarithm of the number of days since administration of the isotope, starting with Day 1.[8,14] The experimental points lie in such a way that a fairly straight line can be drawn through them. The slope of this line can easily be computed and used to describe the steepness of the excretion curve or to compare the rapidity of removal of the isotope from the body. Table 3 summarizes the urinary excretion data for this patient. It should be noted that in all 3 tests the negative values for b (the slope of the excretion curve) were greater than values reported for 2 normal humans exposed accidentally to Sr^{90}.[16] This implies that F.H. was not retaining strontium as efficiently as the normals and is in accord with the observed condition of negative calcium balance, a characteristic of this disease.

ACCRETION RATE

The fund of information concerning the behavior of various isotopes of strontium and calcium in the human body is growing rapidly. The comparative studies of Ca^{45} and Sr^{85} in humans, carried out by the late Dr. Daniel Laszlo and his colleagues, deserve special mention because of their fundamental value.[9] Perhaps the most stimulating suggestions for interpreting metabolic data on bone-seeking radioactive tracers have come from the work of Bauer, Carlsson and Lindquist. They have developed a mathematic relationship between observations of urinary excretion, fecal excretion and serum concentrations, by means of which numerical values can be derived for the rate of calcium accretion and the amount of readily exchangeable calcium in the skeleton.[3] Although several of the assumptions necessary for arriving at the final formulation have not been subjected to rigorous testing by experiment, this method of using excretion data is of great practical value. Table 4 presents the calcium accretion rates for 2 patients, one of whom (R.B.) was receiving treatment for osteoporosis. H C. was hospitalized during healing of a rib subject to recurring fractures. The accretion rate and exchangeable bone calcium are in the normal range for H.C., whereas the low accretion rate for R.B. is the same as that of one osteoporotic patients reported by Bauer *et al.*[4]

SUMMARY

Radioactive strontium can serve as a tracer to gain information concerning calcium metabolism in human subjects. Gamma-emitting Sr^{85} is used rather than the

TABLE 4. EXCRETION OF SR^{85} AND CALCULATED CALCIUM ACCRETION RATES

Patient	Diagnosis	% of Dose of Sr^{85} *in Urine and Stool* During First 5 Days After Injection	Accretion Rate* (Gm. Ca Per Day)	Exchangeable Bone Calcium* (Gm.)
R.B.	Osteoporosis	70 0	0.19	2.7
H C.	(?) Possible osteoporosis or osteomalacia	42.4	0.72	4.8

* Calculated from daily excreta and serum levels of Sr^{85} using the method of Bauer, Carlsson and Linquist.[3]

much more hazardous beta-emitting Sr^{89} and Sr^{90}. (Ca^{47}—the ideal tracer for normal calcium—is quite expensive and difficult to procure.) Very significant information may be obtained merely by measuring and recording the changes in radioactivity in various body areas during the first hour after intravenous injection of the bone-seeking radioisotope. This is accomplished by placing a lead-shielded gamma-scintillation detector in contact with the skin over the sites of interest and recording the activities on a scaler or ratemeter. The activity versus time curves so obtained are called radioisotope osteograms. Data were presented that indicated that Sr^{85} osteograms for patients afflicted with osteoporosis, Paget's disease, tumor metastases to bone and, possibly, multiple myeloma differed significantly from those obtained from subjects with no skeletal abnormalities. Some interpretations of these deviations were discussed. The value of conducting double-tracer tests (for example, Sr^{85} plus radioiodinated serum albumin) was demonstrated, and correlations with excretion data were made With further refinements the technic ultimately may become useful for certain diagnostic problems in the clinic and for evaluating the efficacy of treatment of these disorders.

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Osteogrammas a Radioisotopo — Studios Cinetic de Disordines Skeletic in Humanos

Summario in Interlingua

Strontium radioactive pote servir como traciator in obtener informationes relative al metabolismo de calcium in subjectos human. Le strontium usate es Sr^{85} (emittente radios gamma) e non le multo plus hasardose Sr^{89} e Sr^{90} (emittente radios beta). Le traciator ideal pro calcium normal esserea Ca^{47}, sed illo es multo costose e difficile a obtener. Multo significative informationes pote esser obtenite per le simple mesuration e registration del alterationes de radioactivitate in varie areas del corpore in le curso del prime hora post le injection intravenose del osteotropic radioisotopo. Isto es effectuate per placiar, in contacto con le pelle in le sitos de interesse, un detector de scintillation de radios gamma con armatura de plumbo, utilisante pro le registration del activitate un indicator de radiation a scala calibrate. Le assi obtenite curvas de radioactivitate como function del tempore es designate osteogrammas a radioisotopo. Es pre datos que indica que osteogrammas pro patientes con osteoporosis, mor Paget, metastases maligne al osso, e sibilemente—myeloma multiple differ nificativemente ab osteogrammas a Sr subjectos sin anormalitates skeletic. interpretationes possibile de iste devia es discutite. Es demonstrate le valor d dios bi-traciatori (per exemplo con albumina seral marcate con iodo ra tive). Correlationes con datos de exc es presentate. Il es a previder que r mentos additional va render iste tec utile in le solution de certe problemas nostic in le practica clinic e etiam evaluation del efficacia de mesuras the tic in casos del supra-mentionate disor

16

Quantitation of Mineralization of Bone As an Organ and Tissue in Osteoporosis

JAMES S. ARNOLD, M.D.*

The disease of osteoporosis in humans has long been recognized as a condition in which there are softening and increased radiolucency of the skeleton. By definition, in osteoporosis there is a decrease in mineral content of the skeleton as a whole. However, much confusion has existed as to how the loss of mineral occurs. For many years pathologists have recognized that the softening of bone in osteoporosis is accompanied by a decrease in the amount of bone tissue, both grossly and microscopically. Some observers have been so impressed by the softening of spongy bone that they were led to believe that bone tissue itself must be deficient in its degree of mineralization. To the author's knowledge, no studies have been made to evaluate the degree of mineralization of spongy or trabecular bone, either directly or indirectly, in osteoporosis. All studies made of the ratio of mineral to organic components leave much to be desired, since alterations in the ratio could be due either to changes in the numerator or the denominator, or to both. Studies based on the expression of mineral and organic content per gram of fat-free dry weight are similarly deficient, since here variables (ash and organic matter) are expressed in terms of another variable (dry weight), and the two variables are interdependent. Logically, the variables of composition should be expressed in terms of a nonvariable volume. With this in mind, a method was developed to isolate and measure the volume of trabecular and cortical bone. In the present study, the water and the organic and mineral contents of trabecular and cortical bone have been determined and are expressed in terms of cubic centimeters of hydrated or wet bone for both normal and osteoporotic individuals at autopsy.

Previously little or no effort has been made to quantitate the degree of mineralization of skeletal tissues in order to determine just how much demineralization may have taken place. To add this quantitative aspect to this study, the ash per cubic centimeter of medullary tissue of the lumbar vertebral bodies was determined in each case studied. The vertebral bodies were chosen because they were a readily available source of trabecular bone at the time of autopsy and were thought to reflect easily any change in skeletal mineralization. It was observed histopathologically that trabecular bone rather than cortical bone was affected first and most severely in osteoporosis.

METHODS

In all instances the materials for study were obtained at the time of routine autopsies. The tissues collected were the twelfth thoracic and the first two lumbar vertebral bodies, as well as a strip of cortex from the lateral mid-diaphysis of the femur. The

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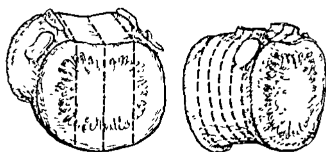


FIG. 1. (*Left*) Diagram of manner of sectioning longitudinally vertebral bodies for study of medullary tissue composition. (*Right*) Diagram of manner of cross-sectioning vertebral bodies for isolation and study of trabecular bone.



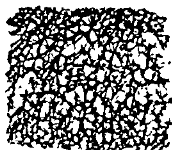


FIG. 2. Sample of trabecular bone used for analysis of composition.

specimens were frozen and stored in the frozen state.

VERTEBRAL MEDULLARY TISSUE ASH/CC. ANALYSIS

The second lumbar vertebral body, while still frozen, was sawn longitudinally in the sagittal plane into slabs 5 to 7 mm. thick, a band saw with a skip-tooth saw tooth blade (Fig. 1, *left*) being used. This saw produces a clean-cut surface with no packing of the saw blade or detectable homogenization of the adjacent tissue. Cutting the tissue in the frozen state prevents the forcing of bone chips into the adjacent tissue. While still frozen, the slabs were placed in cold 4 per cent formaldehyde solution neutralized to pH[9] with sodium hydroxide where fixation takes place. Throughout the process of freezing, thawing and fixation the tissues were held at a constant volume by the included rigid bone structures. Following fixation, all cortical bone and the dense bones of the end plates were removed carefully by sharp dissection. In instances where large herniations of disks or partial collapse of the vertebrae was present, the specimens were discarded. Where the herniations were small, the involved area, together with the surrounding hyperplastic bone, was removed. The cleaned medullary tissue slabs then were placed in cold distilled water and submitted to a vacuum of 5 to 7 mm. Hg for 2 hours, or until all bubbling from the samples had ceased. The vacuum treatment was to remove all entrapped air and the formaldehyde used in fixation. Following return to atmospheric pressure, the specimens were allowed to stand for 1 hour to ensure sufficient time for the water to replace the spaces left by the removal of gas. The specimens next were weighed on an analytic balance while suspended in distilled water at approximately 5° C. The excess water was blotted from the surfaces of the specimens, and they were weighed in weighing vials. Care was taken through the entire processing procedure to keep the specimens cold and, therefore, prevent melting and subsequent loss of included marrow fat. Some specimens were dried and fat was extracted prior to ashing in order to determine the total water, fat and organic content of the marrow. The majority of specimens simply were dried at 100° C. for 24 hours and then ashed in a muffle oven at 580° C. for 48 hours. The resultant ash was weighed and expressed as grams per cubic centimeter of medullary tissue.

TRABECULAR BONE COMPOSITION

The twelfth thoracic or the first lumbar vertebra body, while frozen, was cross-sectioned serially with a band saw into 3 to 4 mm. thick slabs (Fig. 1, *right*), yielding 5 or 6 slabs per vertebral body. The bone marrow included was washed out carefully under a high pressure stream of tap water. Samples to be analyzed were approximately 1.5 × 1.5 cm. squares of trabecular bone cut from the central areas of the slabs. The specimens were degassed in water at a reduced pressure of 5 to 7 mm. of Hg for 45 minutes, and following return to atmospheric pressure they were allowed to stand another 45 minutes in degassed water. They were weighed on a microanalytic balance in distilled water at room temperature while suspended by a fine wire and then were freed of excess water by centrifuging at 8,000 g. for 15 minutes in a refrigerated centrifuge. Next they were transferred to weighing vials and weighed on a microanalytic balance to the nearest .01 mg. This weight after centrifuging was taken as the wet weight of the specimens. The appearance of a cleaned trabecular bone sample is demonstrated in Figure 2.

The difference between weights in water and wet weights (loss of weight in water) by Archimedes' principle is equal to their volumes (assuming the density of water to be unity). A series of experiments on the time and the centrifugation force necessary to remove varying amounts of water from trabecular bone will be published elsewhere. These experiments indicate strongly that only surface water is removed in the conditions prevailing.[3] Following centrifugation the specimens were dried for 4 days, or until constant weight was attained, at 90° C. over anhydrous $CaSO_4$. They were allowed to cool in a desiccator over concentrated H_2SO_4 and weighed in appropriate vials, after which fat was extracted by refluxing in a Soxhlet apparatus using ether-alcohol.*

After extraction, it was found to be necessary to dry for the same extended periods at 90° C. as were used to obtain the original dry weights in order to obtain constant weight. Presumably this was due to the fact that the specimens were rehydrated partially in the process of fat extraction. The small differences between the dry and the extracted weights (usually about .1% of the dry weight) were attributed to fat adsorbed from the bone marrow in the isolation procedure. The specimens then were ashed in a well-ventilated muffle furnace for 48 hours at 570 to 590° C., allowed to dry in a desiccator over concentrated H_2SO_4 and weighed on a microanalytic balance in appropriate vials.

The values for the loss of weight of the dry fat-free specimens in the process of ashing are referred to here as the organic plus volatile inorganic mass. As pointed out by Robinson,[8] in the process of ashing bone in addition to oxidizing and driving off the organic material a variable amount of chloride, potassium, intracrystalline water and, most important of all, CO_2 is lost. By ashing to constant weight continuously at 580° C., which is below the temperature or recrystallization of hydroxyapatite, these volatile fractions of mineral can be separated from the nonvolatile mineral or ash.[8,6]

CORTICAL BONE

The specimens of cortical bone were processed in the same manner as the trabecular bone with a few slight differences. In the isolation of the cortical bone specimens, the periosteum was carefully scraped free of all soft tissue. The strip of cortex was cross-sectioned into multiple 3 to 4 mm. thick slabs. The larger resorption cavities and holes were probed carefully, and the contained soft tissue was removed with probe or forceps. The specimens were washed thoroughly in a fine high pressure stream of tap water to remove as much as possible of the fat and the soft tissue from the larger central canals of haversian systems. How-

* Extraction mixture composed of 7 parts ether and 3 parts 95 per cent ethyl alcohol

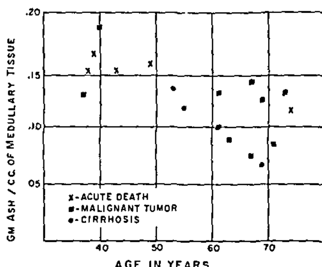


Fig. 3. Ash content per cc. of vertebral body medullary tissue versus age.

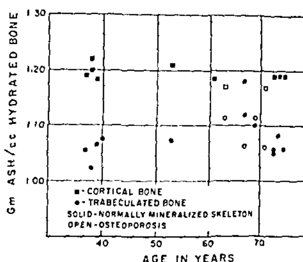


Fig. 4. Ash content per cc. of hydrated trabecular and cotical bone versus age.

ever, under the dissecting microscope, many of the smaller canals still contained some fat and blood vessels that were not removable in the washing procedure. The trabecular bone on the medullary surfaces of the specimens from older individuals and osteoporotics were left attached to compact bone but were cleaned carefully of adherent soft tissue. In the process of drying at 90° C. it required 6 days rather than the 4-day period for trabecular bone samples to attain constant weight.

RESULTS

Vertebral Medullary Tissue Ash/cc.

The values for ash content per cubic centimeter of vertebral body medullary tissue are presented as a function of age in Figure 3. It is unfortunate that the study could not be restricted to individuals dying acutely of traumatic causes. The laws governing the conduction of coroners' autopsies in the State of California prohibit the use of autopsy material for such special studies; consequently, the studies were made on the bone of patients dying of acute causes, such as brain abscess and myocardial infarction, as well as of a variety of tumors Of the tumor cases, only those in which the skeleton both grossly and microscopically was free of metastasis were included in the study. A few of the patients were chronically ill for a period of a year or more prior to death. No cases of endocrine, renal or other disease known to affect the skeleton have been included in these data. It is apparent from the data that there is a definite trend toward a decrease in the amount of mineral (and, as will be seen, bone tissue) as a function age between 37 and 75 years of age. Pathologically recognizable osteoporosis was apparent in all cases containing less than 0.10 Gm. of ash per cc. of medullary tissue. Therefore, it is apparent that cases of pathologically recognizable osteoporosis contained from one half to one third of the mineral content of younger controls and approximately one half that of controls of comparable age. The small numbers involved in the various types of patients studied prevent any further interpretation of the data.

Bone Composition

The data for ash per cubic centimeter of hydrated bone tissue are presented graphically as a function of age in Figure 4. It is apparent that the cases of osteoporosis studied here have comparable or slightly higher values of ash per cubic centimeter of hydrated trabecular bone than do the controls

of comparable age. It is also apparent that the degrees of mineralization of three of the four cases below 40 years of age are lower than the values seen in the older individuals.

While the degree of mineralization of trabecular bone tends to increase as a function of age, it is characterized by varying over a range of 5 per cent from case to case. On the other hand, the degree of mineralization of cortical bone shows no tendency to change as a function of age and shows much less variation from case to case. The standard deviations for the ash per cubic centimeter of hydrated bone measurements of trabecular and cortical bone were 0.5 and 0.2 per cent, respectively, which is contributed almost entirely in each case by the error of determining the volume of the hydrated specimens. The results indicate that cortical bone contains from 10 to 15 per cent more mineral per unit volume than does vertebral trabecular bone.

The more detailed compositional data are presented for 10 cases in Table 1 and include water and organic plus volatile inorganic and ash contents per cubic centimeter of hydrated bone, as well as hydrated density. In each case these values are the average obtained from four samples. On inspection of the hydrated density values for trabecular bone, it is apparent that these values are lower in the younger adults and higher in the older group. It is apparent too that the cases with low hydrated densities also have low ash contents and vice versa. This indicates that the density values are simply a reflection of the mineral content, which, of course, they should be, since the density of ash is almost 3.2 times that of water. In all cases the fat content of the specimens was small, representing from .0 to 1.5 per cent of the hydrated weights of the specimens. In each case this fat was considered to be a contaminant; however, the volumetric data presented have not been corrected for its presence. The mass of fat in individual cases can be calculated readily as the difference between the sum of the listed constituents (water and organic plus volatile inorganic and ash contents/cc.) and the hydrated density.

When the concentrations (mass per unit volume) of ash and water in Table 1 are compared, it is apparent that the cases containing low ash content have high water content and vice versa. This would suggest that

VOLUMETRIC COMPOSITION OF TRABECULAR AND CORTICAL BONE

	Case No.	Age Yrs.	Hydrated Density	H_2O/cc.	Organic plus Volatile Inorganic/cc.	Ash/cc.
Trabecular						
	1	38	1.886	.2919	.5555	1.028
	2	39	1.894	.2801	.5524	1.061
	3	40	1.929	.2722	.5751	1.077
	4	53	1.934	.2700	.5709	1.073
	5	61	1.956	.2529	.5719	1.116
	6	67	1.928	.2667	.5781	1.070
	7	69	1 946	.2591	.5555	1.116
	8	73	1 911	.2669	.5728	1.062
	9	74	1.930	.2673	.5713	1.087
	10	75	1.921	.2747	.5737	1.069
Cortical						
	4	53	2 011	.2278	.5550	1.212
	5	61	1.967	.2388	.5577	1.144
	10	75	1.996	.2443	.5504	1.191

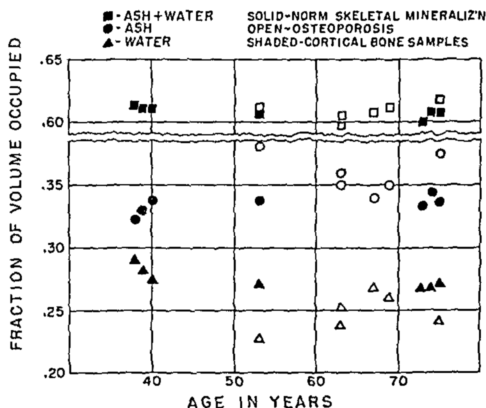


FIG. 5. Fractions of the wet volume occupied by water and ash (nonvolatile mineral) and their sums versus age.

the increased mineral deposited in bone replaces water or, when removed, is replaced by water. In order to represent this volumetric situation graphically, the fractional volumes occupied by the ash and the water are plotted together as a function of age (Fig. 5). The volume of the ash is found by dividing its mass by its experimentally measured density, which was found to be 3.181 ± .02.[6] When the sums of the fractional volumes occupied by ash and water thus are plotted against age, they are found to be quite constant, occupying from 0.600 to 0.615 of the volume of the hydrated trabecular bone. When the ash is high, it is clear that the water is decreased to precisely the amount necessary to accommodate the volume of the increased mineral and vice versa. There may be a slight negative slope to the line of sum of the ash and the water volumes in trabecular bone as a function of age, indicating that some other component such as volatile inorganic material or (less likely) organic material may be increasing with age to occupy a progressively larger fraction of the volume of the hydrated trabecular bone More data will be necessary to establish the reality of the present suggestion of a negative slope. It appears clear that the ash plus water fractional volume is not altered in the three cases of osteoporosis included in these data.

It is also to be noted in Figure 5 that the fractional water plus ash space of the three samples of cortical are quite similar to the corresponding values for trabecular bone. The increased fractional volume occupied by the additional ash content of cortical bone is accompanied by a corresponding decrease in the fractional water volume.

The combined mass of organic plus volatile inorganic content per cubic centimeter of hydrated bone is plotted as a function of age in Figure 6. This combined mass has a value of 0.55 to 0.56 for the three samples of cortical bone and trabecular bone of the two younger adults dying acute deaths. The trabecular bone samples of the remainder of the older individuals have, with one exception, organic plus volatile inorganic values of 0.57 to 0.58. Of the older individuals, three were osteoporotic, two of whom had organic plus volatile inorganic contents of 0.57 to 0.58, and the third had less. At the present

time it is impossible to interpret the significance of these data, since it is not known whether the organic or the volatile inorganic, or both, are varying. Since the CO_2 content of bone is known to increase with age and is a potentially more labile component, it appears likely that those variations are due to changes in volatile inorganic content. It is difficult to visualize how osteocytes entrapped in their lacunae could deposit organic material actively. Studies of the direct and separate analysis of both organic and volatile inorganic content are being conducted to answer this question. In the interpretation of the organic plus volatile inorganic values it should be pointed out that these values contain the maximal analytic error encountered in this study. Their probable errors are the summation of both the errors in obtaining the true dry fat-free and ashed weights of the specimens, as well as the error in the hydrate volume measurement. The standard deviation of sets of four presumably identical specimens processed together was ±.002 or 0.4 per cent. Separately processed and more randomly selected samples of trabecular bone from the same case yield a standard deviation of 1.2 per cent in the organic plus volatile inorganic values. The differences noted from case to case clearly are greater than can be accounted for on the basis of analytic error.

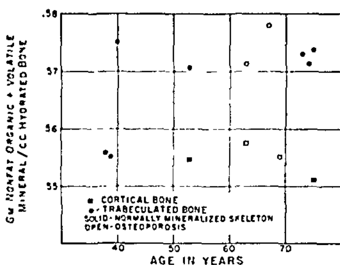


FIG. 6. Mass of organic plus volatile inorganic material per cc. of hydrated trabecular bone.

DISCUSSION

The results of the quantitation of mineral content within vertebral bodies indicate that the mineral content of this more labile skeletal component must be reduced to about half its normal values to become recognizable, both pathologically and clinically, as osteoporosis. Values less than one third that of normal young adults were not seen in the four cases studied.

The composition of the trabecular bone in the three straightforward senile or idiopathic osteoporotics is indistinguishable from that of controls of comparable age and normal degrees of vertebral mineralization. The values for organic plus volatile mineral found per cubic centimeter of hydrated bone in the trabecular bone of the osteoporotics were comparable with those of controls. According to the concept introduced by Albright and Reifenstein[1] that an insufficiency of organic matrix formation is the basic defect in osteoporosis, one might anticipate a decreased amount of organic material. Such a change is not present in our data. It has been demonstrated by microradiographic technics that cortical bone is mineralized slowly but progressively following deposition from about 80 per cent of its maximal mineralization soon after deposition to full mineralization.[2] Assuming that the same process occurs in trabecular bone, the deposition of new bone should add poorly mineralized bone to the trabeculae that increase progressively in its mineralization. If the rates of deposition and resorption are equal, the rate of new bone formation should be related directly to the mean age of the bone and, therefore, related inversely to the average mineral content. Following this line of reasoning, it would appear that the rate of bone deposition in osteoporosis is comparable with that present normally in older individuals. This is in good agreement with the findings that the

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Skeletal Distribution of Estrone-16-C^{14}*

ANN M. BUDY, PH.D.†

Studies with radioactive estrone have been complicated by (a) marked species difference in the metabolism of and reactivity to hormones[2,12] and by (b) the fact that the distribution and the localization of the steroids appear to be quantitatively more closely related to their metabolism, and to their excretion and that of their metabolic products, than to their specific affinity for the target organs of their physiologic activity.[4,5] Bone is a target organ characteristic for the mouse. Evidence for this is based on the production of endosteal bone by the mouse under the influence of estrogen and on the localization of radioactivity in bone following administration of radioestrone at a time when other tissues have become free of this activity. For example, following a single dose of 0.1 mg. (0.27 μc.) of estrone-16-C^{14}, radioactivity is present in both uterus and bone, but it disappears quickly from the uterus while remaining for a much longer period in bone. The appearance of activity in a target organ can be assumed to take place rapidly; the longer persistence in bone suggests a more prolonged action in this tissue. It is proposed further, from results of previous experiments and from those reported here, that there is an interaction between the estrogen and/or its metabolites with some cellular fraction; e.g., bone marrow and bone cells.

MATERIALS AND METHODS

Animals. Mice of the CF_1 strain, 21 to 40 days of age, weighing 16 to 21 Gm., were used. The ages and the weights are specified in the descriptions of the experiments below. All mice were killed by cervical fracture. The heart was exposed by removing the sternum, and blood was withdrawn immediately by cardiac puncture.

Estrogens. Carrier-free estrone-16-C^{14} (m.p. 261-262° C.; rotation +161°) having a specific activity of 2.7 μc./mg. was purchased from Charles E. Frosst and Company, Montreal. For intramuscular injections the radioactive estrone was dissolved in sesame oil in a dilution of 1 mg/ml. A dose of 0.1 mg. of radioestrone was calculated to have 306,000 disintegrations per minute. When larger doses were used (1.0 mg.) the radioestrone was dissolved in ethanol and given intraperitoneally.

Estrone (nonradioactive) (m.p. 254-259.8° C.; rotation +162°; Kober 98%) was supplied through the courtesy of Ayerst, McKenna and Harrison, Ltd. The crystalline hormone was dissolved in sesame oil for intramuscular injections of 0.1 mg., in propylene glycol for doses of 0.5 mg., and in ethanol for doses of 1.0 mg, given intraperitoneally.

Estimation of Respiratory CO_2. Following administration of radioestrone, the mice were placed in individual glass metabolism cages designed to collect expired CO_2 in towers of NaOH and to collect urine and

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feces separately.[9] Methods for the determination of expired CO_2 in mice of Group I were reported previously.[4] In mice of the other groups, expired CO_2 was determined by a modified Van Slyke wet combustion method.[13] The CO_2 was placed in an ionization chamber,[3] and the charge in the chamber was measured with a vibrating reed electrometer (Applied Physics Corp., Pasadena, Calif.; Model No. 30). Collections and determinations of expired CO_2 were made on all mice daily so long as radioactivity could be detected in urine and feces.

Estimation of Radioactivity in Tissues and Fluids. Radioactivity in all fluids and homogenized tissues was counted in windowless gas-flow counters operating at a counter efficiency of 51 ± 1 per cent.[4] For determinations of total carbon and radiocarbon, the methods of Van Slyke and coworkers[14,16] were used.

Extraction Technic and Paper Partition Chromatography. The technics used for extraction of steroids from urine and bone and for the chromatographic procedures were those published in a previous report.[4] Urine was collected from all mice following injection and during the experimental period.

Autoradiography.* Undecalcified femurs were embedded in paraffin and trimmed longitudinally by the method of Lotz *et al.*[8] The cut bones in blocks were placed first in direct contact against Kodak No Screen X-ray film and then in a container and stored at low temperatures during exposure.

Microradiography.† Femurs and tibias of an estrogen-treated and an oil-treated mouse were fixed in absolute alcohol, dehydrated with ether, embedded in methyl methacrylate and cut undecalcified with a high-speed rotating saw into cross sections. The serial sections were cut at about 4 mils (0.1 mm.) with a loss of 6 mils (0.15 mm.) between the sections. The sections were first made in the center of the shaft, then advanced in 10-mil (0.25 mm.) steps toward the epiphyses. The microradiograms were made by pressing the bone sections into contact with Eastman Kodak Spectroscopic Plate 694-0 and then by exposure to a beam of 10 kv. x-rays at 20 ma. for 10 minutes.

Measurement of the Osteogenic Reaction. With a dose of 0.5 mg. of estrone weekly, about 12 weeks was required for obliteration of the marrow cavity of the femur.[11] The reaction, from early changes to complete obliteration, has been divided into 4 stages from 1+ to 4+, and a skeletal reaction of 1+ to 2+, demonstrable by roentgenograms within 4 weeks, has been the usual end point in our experiments.

With a dose of 0.5 mg. of estrone weekly, osteoblastic reaction in the submetaphysial area could be demonstrated histologically as early as 4 days after injection, and endosteal bone was seen within 7 days. Increased density in the metaphysis could not be demonstrated roentgenographically in less than 10 days but subsequent to this afforded a convenient method of following the progress of the skeletal reaction.

EXPERIMENTAL

Except when it was desired to introduce the maximum amount of radioactivity into the animal, either to demonstrate that the radioestrone produced the same reaction in the bones as that produced by estrone (nonradioactive) or to provide optimum conditions for counting or for autoradiography, the plan followed in most experiments was to administer estrone (nonradioactive) and radioestrone (estrone-16-C^{14}) simultaneously. The purpose of this procedure was to ensure an optimum dose of the hormone for the effect that it was desired to produce upon bone while limiting the radioactivity to that necessary for the tracer effect. In one

* The autoradiograms were made by Dr. W E. Lotz, at Oak Ridge, Tenn

† The microradiograms were made by R. E. Rowland, at Argonne National Laboratory, Lemont, Ill.

group (No. 4) the animals were primed with estrone for 4 weeks in order to obtain a maximum effect on the bones before the tracer radioestrone was administered.

Group 1.* Twelve female mice, 40 days old and weighing 16 to 18 Gm., were injected intramuscularly with 0.1 mg. of radioestrone in sesame oil. Two mice each were sacrificed at intervals of 6, 12, 24, 48, 72 and 96 hours after injection. Two additional mice were given the same dose of radioestrone; urine and feces were collected, and radioactivity was determined daily. At 144 hours, when excreta were free of radioactivity, these mice were killed; only trace amounts of radioactivity were present in liver and bone.

Group 2. Two mice, male and female, 40 days old and weighing 18 Gm. each, were injected intramuscularly with 0.1 mg. of radioestrone in sesame oil every 3 days over a period of 15 days. Two additional mice were given the same volume of sesame oil and were used as controls. The mice were killed 1 week later; endosteal bone formation was demonstrable in roentgenograms.

Group 3. Eight mice, 4 males and 4 females, 23 days old and weighing 18 to 21 Gm., were each injected with 0.1 mg. of radioestrone in 3 doses on Days 1, 8 and 15 and 0.2 mg. of estrone on Days 4 and 11. The hormones then were discontinued. Two additional mice, male and female, were given the same volume of sesame oil and were used as controls. The mice were killed at 3, 4, 6 and 7 weeks after the last dose of radioestrone. Only those organs in which radioactivity could be detected 3 weeks after the final dose were prepared for study. Whole-body roentgenograms were taken at autopsy. After radioactivity was determined in the homogenized bone samples the remaining homogenates of all bone specimens in Groups 1, 2 and 3 were pooled and evaporated to dryness for extraction.

Group 4. Twelve mice, 6 males and 6 females, 40 days old and weighing 18 to 19 Gm., were divided into 3 subgroups. One subgroup each received 0.5 mg. of estrone in propylene glycol weekly for 4 weeks, then 1.0 mg. of estrone in ethanol intraperitoneally. The second subgroup each received 0.5 mg. of estrone in propylene glycol weekly for 4 weeks, then 1.0 mg. of radioestrone in ethanol intraperitoneally. The third subgroup received the same volume of propylene glycol (0.05 ml.) weekly for 4 weeks and the same volume of ethanol intraperitoneally. Roentgenograms were taken of each mouse just before each injection every week. All mice were placed in individual glass metabolism cages for collection of expired CO_2 and urine, and were killed 3 to 5 hours after the intraperitoneal injections. The sternums were removed, and the specimens were trimmed so that 2 mm. of costal cartilage remained attached. The specimens then were dried at room temperature and prepared for combustion.

Femurs and tibias were dissected out, blotted free of blood, and weighed and placed in formalin for 48 hours. Then they were washed in distilled water and dried at room temperature. Under x-ray control, the upper and the lower ends of femurs and tibias were cut so as to include the elongated metaphyses within the end portions, leaving the shafts free of gross medullary bone.

RESULTS

The osteogenic effect of radioestrone alone is illustrated in Figure 1. Density can be observed in the tibial metaphysis, in the distal end and along the endosteum of the femur. The intramedullary reaction is identical with the skeletal response produced with nonradioactive estrone.

The results observed in mice receiving 0.5 mg. of radioestrone in 3 weeks indicate

* Except for the detailed results on bone, the distribution of radioestrone in tissues of this group has been reported previously.[4]



FIG. 1. Roentgenograms of femurs and tibias of mice from Group 2 taken on the 21st day of the experiment. (*Left*) Control. (*Right*) Experimental. Total dose of 0.5 mg. of radioestrone in 15 days. Note the endosteal new bone formation in the proximal end of the tibia, the distal end and along the endosteum of the femur. (× 5)

the relationship between the dose of the estrogen and the skeletal response; the magnitude of bone response and the rate of endosteal new bone formation are proportional to the dose of the hormone and the duration of the treatment.

The retention of radioactivity in bone following single and repeated doses of radioestrone is illustrated in Figure 2. The total radioactivity measured in bone represents that due to radioestrone and/or its metabolites that remain labeled with C^{14}. However, since the metabolites have not been characterized, the data are reported in terms of microgram equivalents of injected estrone. The first 6 bars represent the radioactivity in bones of mice of Group 1 and the remaining 4 bars are from Group 3.

From 3 to 4 weeks after a total of 0.3 mg. of radioestrone and 0.4 mg. of carrier estrone had been given, no radioactivity was detected in liver, spleen, lung, testes, ovaries, uterus, bile, feces or urine. There were trace amounts in blood and in adrenals. Significant counts were obtained in bone, axillary lymph nodes, muscle and skin at the site of injection, and the remaining carcass.

At 5 to 6 weeks with the same dosage, there were traces of radioactivity in the lymph nodes and significant counts in bone, but none in any other tissue. The density increased with repeated doses, and, when administration of the steroid was discontinued, there was a decrease in the density of bone in the metaphyses. With multiple doses the level of retention of radioestrone increased gradually and was maintained at the higher level for an additional 3 weeks.

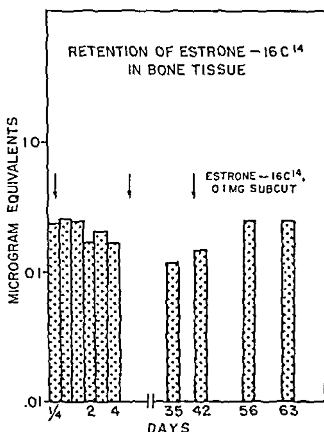


FIG. 2. Retention of radioestrone in bone. Arrows indicate the time of administration. The amount of radioactivity detected in bone is plotted on semilog paper as microgram equivalents of injected dose per gram tissue used in the determination. A microgram equivalent is defined as the tissue radioactivity equivalent to 1 mcg. of labeled estrone injected; as a matter of convenience it is expressed as μg./Gm.

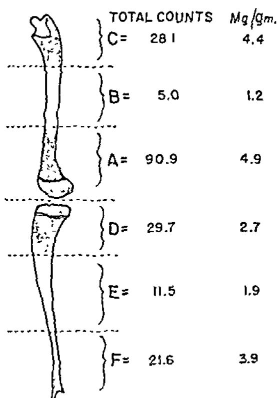


FIG. 3. Diagram of a mouse femur and tibia of Group 4, drawn and enlarged from roentgenograms of 8 mice taken after 4 weeks of administration of 2 0 mg. of estrone. The density of the bones in the roentgenograms is indicated by the stippled areas on the diagram. Total counts represent the total (2 mice) amount of radioactivity found in the various segments of bone 3 to 5 hours after intraperitoneal injection of 1.0 mg. of radioestrone. (× 3)

Roentgenograms of the mice at 56 and 63 days indicated that the subepiphysial area had decreased in density; the new bone that had been formed under the influence of estrogens was being resorbed, and only a faint reaction could be detected, although the level of radioactivity was maintained. Normal bone growth reappeared just below the epiphysial cartilage plate.

The autoradiograms of the bones from mice given single doses of ... were negative. This was to be ... only 0.27 μc. of the ... ed, and only a very small ... was concentrated in bone. A

a mouse of Group 2 receiving 0.5 mg. (1.35 μc.) of radioestrone indicated some activity along the endosteum, some in the bone marrow and a slight outline at the site of the epiphysial plate. Since it was necessary to expose the film for 10 months, there was consid... fogging.

T... of these experiments not only ... ious observations of the skeletal ... estrogens in mice but also ... rent approach to the study of ... In Group 4 ... manometric ... carbon

and its radioactivity in blood, bone, sternum and urine was used. This method is more sensitive than the plate-counting method of homogenates used for Groups 1 to 3. All mice showing a 2+ endosteal bone reaction were sacrificed within 3 to 5 hours after the final intraperitoneal administration of the steroid. The amount of radioactivity detected in the different segments of bone is illustrated in the diagram of Figure 3. The segments of the femurs and the tibias of 2 mice were pooled for these observations. The amount of radioactivity detected in the shafts was to have been expected, since these animals were under estrogen treatment for 4 weeks and there was evidence of an endosteal reaction in the shafts. At this stage of medullary bone formation there are numerous discrete areas of bone marrow and vascular sinuses surrounded by the new bone.

Carbon and radiocarbon determinations of bone, blood and urine in mice of Group 4 are presented in the following table. Evidence for the rapid absorption and excretion of radioestrone and/or its metabolites is the high levels of radioactivity in blood and urine following intraperitoneal injection.

Figure 4 illustrates microradiograms of cross sections of a femur of an estrogen-treated mouse and of a tibia from a control mouse, both from Group 4. Figure 4, *left*, is a section taken approximately 2 mm. below the cartilage plate. A core of bone marrow is present in this area, whereas the area immediately below the cartilage plate (not shown) is filled completely with endosteal bone. Figure 4, *center*, is taken from the shaft of the femur; the roentgenograms give the appearance of denser cortical bone with slight irregularity in the density along the endosteum. The endosteal reaction in the shaft may explain the radioactivity found in these segments. Higher magnification of

Carbon and Radiocarbon Determinations in Estrogen-Treated Mice

Sample	Treatment and No. of Mice		Total Dry Wt. (mg.)	Total Carbon mg. (corr.)	Counts/mg. Carbon (corr.)	Total Counts	dpm/Gm.	µg./Gm.
Femur	C	(2)	68.2	12.2	—	—	—	—
	E	(2)	81.4	11.4	—	—	—	—
	E + R	(2)	95.1	18.2	18.3	124.0	3,214	10.5
Tibia	C	(2)	67.1	11.2	—	—	—	—
	E	(2)	65.3	10.3	—	—	—	—
	E + R	(2)	73.2	12.4	14.7	63.0	2,627	8.5
Sternum	C	(2)	74.3	16.6	—	—	—	—
	E	(2)	108.2	25.8	—	—	—	—
	E + R	(2)	49.2	16.5	20.3	164.0	3,325	11.0
			Vol. (ml.)					*µg./ml.*
Blood	C	(4)	0.1	14.7	—	—	—	—
	E	(4)	0.1	12.9	—	—	—	—
	E + R	(4)	0.1	8.7	140.9	1,221.0	12,213	399.1
Urine	C	(4)	0.1	3.2	—	—	—	—
	E	(4)	0.1	3.3	—	—	—	—
	E + R	(4)	0.1	3.5	1,508.0	5,273.0	52,730	1,723.0

Mice from Group 4. C = control, E = nonradioactive estrone; E + R = nonradioactive estrone + estrone-16-C14. The numbers are given to the nearest decimal point. *Bone samples:* data from mice killed 4 hours following intraperitoneal injections. *Blood and Urine Samples:* data from all mice killed from 3 to 5 hours following intraperitoneal injections. Determinations were made on pooled samples within each subgroup. (See Fig. 3 for distribution of radioactivity in segments of femur and tibia.)



FIG. 4. Microradiograms of cross sections of femur and tibia of mice from Group 4. (*Left*) Section from the metaphysis of a femur of an estrogen-treated mouse approximately 2 mm. below the epiphysial cartilage plate. (× 50) (*Center*) Section taken from the shaft of the same femur slightly below the midpoint of the shaft. (× 70) (*Right*) Section taken from the tibial shaft of a control mouse. (× 60)

the microradiograms of these sections indicated that the newly formed bone was disorganized and consisted of both nonhaversian bone and primitive haversian systems with a suggestion of lamellar structure. The section of the tibia in Figure 4, *right*, illustrates the smooth endosteal surfaces in the control mouse.

Identification of fractions from homogenized bone extracts and from urinary extracts yielded the same results as those previously published.[4] Identification was based on qualitative tests, chromatograms and their autoradiograms. It was observed that the radioactive fractions from bone extracts consisted of free steroids, estrone and estradiol-17β, an ethereal sulfate conjugate and a glucuronide conjugate. A positive test was produced with the residues of both urine and bone in the presence of diazotized sulfanilic acid.

No measurable radioactivity was found in the expired carbon dioxide at any interval from 3 hours to 144 hours after administration of radioestrone; there were significant counts in the lungs up to 12 hours.

DISCUSSION

Medullary bone formation in mice from administration of estrogen does not reflect any known physiologic function. There are certain limitations with the use of labeled estrogens. For example, radioactivity alone is measured, and this represents carbon-16 of the molecule and not estrone itself, so that the presence of radioactivity does not give any information on the intermediate steps in metabolism of the steroid Qualitative tests were used on chromatograms of the urinary and bone extract fractions, but the identification of metabolites must remain on a tentative basis until sufficient material is obtained for quantitative tests and for characterization of the steroid and its metabolites. Despite these limitations, some over-all information with respect to metabolism and the excretion of the steroid was obtained. There was no difference between the male and female in metabolizing the hormone, nor was there any difference in new bone formation.

It was assumed early in the study that the deposition of C^{14} would be demonstrated in

the form of carbonate in bone. Experimental results reported by others and in this study demonstrate that this does not occur. No one has been able to show autoradiographically the uptake of C^{14} from radioestrone in cortical bone. Twombley and Schoenewaldt could not demonstrate the incorporation of C^{14} from labeled diethylstilbestrol into the mineral of bone in mice, although the synthetic estrogen produces a good osteogenic response.[10] Leblond[7] found no evidence of radioactive carbon in the bones of mice treated with radioestrone. There is no reason to believe that the carbon ring D of estrone is sufficiently labile to enter into reaction in the form of carbonate of the bone. This is confirmed by the fact that no measurable radioactivity could be detected in the expired carbon dioxide.

From these results it is apparent that the emphasis on C^{14} detection must be shifted away from bone mineral to the bone marrow and the cellular elements of bone. Based on histologic evidence it had been suggested[11] that the cellular elements participate actively in the osteogenic reaction; it was felt that estrogen-induced bone changes are a simple effect upon the osteogenic connective tissue of the primitive bone marrow. Since radioactivity is detected in bone as early as 4 hours after administration, the uptake by bone can be assumed to take place rapidly. This is strongly suggestive of an interaction between the steroid and its metabolites with some cellular fraction. Complete evidence for this is lacking owing to technical difficulties. Gillette and Buchsbaum have reported[6] the results of a study of the response of connective tissue cells in vitro to steroid hormones. They obtained mouse fibroblasts from explants of embryonic long bones and studied the response of cells in a perfusion apparatus. Estradiol had no direct effect on fibroblasts. It was demonstrated by Urist, Budy and McLean[11] that mice under 14 days of age were unable to produce endosteal bone.

In a current review, Villee presents evidence and speculation regarding the mode of action of estrogen at the cellular level. The mechanisms of hormone action may involve enzyme systems in various ways to produce a response within a specific target organ.[17] It is conceivable that such a system operates in bone, although there is little supporting evidence for this. The mechanisms involved from the time of administration of the estrogen to the demonstrable changes in the endosteum are unknown. These mechanisms can include substances or agents that will stimulate the proliferation of osteoblasts in discrete areas in the endosteum.

The response of mouse bone to estrogens is unique among the mammals studied thus far. The most important contribution gained from studies with radioactive hormones is the repeated emphasis and confirmation of the marked differences in species and strain in the metabolism of the hormone. For example, Barry and co-workers investigating the fate of radiotestosterone and radioprogesterone in mice and rats reported that the metabolic transformation and disposal of the labeled hormones were totally different from those of man. Furthermore, even closely related species, as mice and rats, eliminate metabolites of the hormones in dissimilar fashion.[1] The difference in metabolism of estrogens might account for the different effects on bone; a mouse will produce endosteal bone, a rat will inhibit resorption of spongiosa, the hamster will produce fibrous changes in the bone marrow, and the rabbit will show less cortical bone than normals. Since the effects of estrogens in mice in no way resemble those in other mammals, there is no justification for extrapolating the data to man.

SUMMARY AND CONCLUSIONS

Estrone-16-C^{14} was found to possess osteogenic properties *identical* with those of nonradioactive crystalline estrone. Using tracer methods, these studies afford some understanding of the interaction between the steroid and its *metabolites* and the skeletal

response. Additional evidence gained from these studies is as follows:

1. Within 6 hours of a single dose of radioestrone, significant amounts of radioactivity can be detected in homogenized bone; there is no evidence for elimination of radioactivity in bone samples during any time interval up to 96 hours.

2. Evidence is presented that the labeled carbon from Ring D of estrone does not enter into the reaction in the form of carbonate of bone. Activity appears to be concentrated along the endosteum, which is the site of first reaction to estrogens, rather than in cortical bone.

3. Identification of fractions from homogenized bone extracts was based on qualitative tests, chromatograms and their autoradiograms. Tentative identification of radioactive substances is as follows: free steroids, estrone and estradiol-17β, an ethereal sulfate conjugate and a glucuronide conjugate. A positive test was produced with bone residue in the presence of diazotized sulfanilic acid.

4. The evidence from tracer studies with estrone-16-C^{14} is that, in the mouse, estrogen and/or its metabolites act on the bone marrow and cellular elements of bone.

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Le Distribution Skeletic de Estrone-16-C^{14}

Summario in Interlingua

Le distribution e le localisation de estrona radioactive pare, in terminos quantitative, esser relationate plus intimemente a su metabolismo e al excretion de illo mesme e de su productos metabolic que al affinitate specific de su activitate physiologic pro certe seligite organos. In le caso del mus, osso es un tal organo. Iste assertion es basate super le observate production de osso endosteal per muses que se trova sub le influentia de estrogeno e le localisation de radioactivitate in le ossos de muses a un tempore post le administration de radioestrona quando altere tissus es redevenite libere de tal activitate. Es presentate indicios que pare demonstrar que le radiomarcate carbon de anulo D de estrona non entra in le reaction in le forma de carbonato de osso. Le activitate es concentrate apparentemente al longo del endosteo (que es le sito del prime reaction a estrogenos) e non in osso cortical. Studios con estrona-16-C^{14} como traciator indica que, in muses, estrogeno e/o su metabolitos affice le medulla ossee e le elementos cellular de osso. Proque le effectos del estrogenos in muses es non del toto simile a lo que ha essite observate in altere mammales, nos ha nulle justification de applicar le presente constatationes al homine.

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The Influence of Alterations in Parathyroid Function on the Distribution of Plasma Calcium*

SMITH FREEMAN AND MOIRA BREEN†

The distribution of plasma calcium into a minimum of three fractions has been clearly established. Greenberg and collaborators[20] found that approximately 60 per cent of the plasma calcium was ultrafilterable, the remainder being bound to the plasma protein, particularly albumin. Similar[16] or somewhat higher[23] values have been obtained by others using ultrafiltration technics. Earlier, Rona and Takahashi,[18] using compensation dialysis, found the diffusible calcium to be approximately 65 per cent of the total. The classic studies of McLean and Hastings,[12] using the frog-heart technic for Ca^{++} estimation, made evident that the Ca^{++} concentration of plasma normally was slightly less than the value for total ultrafilterable calcium. Studies on the in vitro complexing of calcium by citrate by Shear and Kramer,[21] Hastings, McLean and collaborators[8] and others indicated that a small portion of the diffusible fraction normally was present as the citrate complex. According to McLean, Barnes and Hastings,[11] the fraction of the total plasma calcium that is ionized appeared to be constant over a considerable range of calcium concentration varied by altering parathyroid function. This finding was in agreement with the measurements by Greenberg and Gunther,[6] which indicated that the binding capacity of plasma albumin for calcium was far from saturated under normal conditions and not likely to be exceeded by hypercalcemias encountered in pathologic states. The nature of the assay procedure required for estimation of Ca^{++} concentration by the frog-heart method discouraged its use by many investigators interested in calcium metabolism. However, studies by McLean and collaborators provided a basis for calculating the Ca^{++} concentration of plasma.[13] Differences in the binding of calcium by different plasma proteins and the possibility of colloidal calcium phosphate being formed under certain conditions[7] limited the usefulness of prepared nomograms as applied to conditions in which the composition of plasma may be altered considerably from normal in its protein, phosphate or citrate content.

Studies of the state of calcium in body fluids received additional impetus in 1951, when Raaflaub[17] reported a chemical method of Ca^{++} estimation that gave normal values for plasma Ca^{++} concentration similar to those obtained by the frog-heart method. This procedure has been modified by Rose[19] and applied by Fanconi and Rose[4] to the study of normal subjects and

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patients with diseases in which the concentration or state of calcium might be expected to vary from the normal. The sensitive analytic technics now available for estimating calcium should make possible more critical experimental and clinical investigation concerning the state of calcium in body fluids. The experimental study to be described was undertaken to determine whether or not variations in parathyroid function had any effect on the distribution of calcium in plasma, the ultracentrifuge being employed as a means of separating the plasma calcium into protein-bound and nonprotein-bound fractions.

LABORATORY METHODS AND ANIMAL PROCEDURES

Total calcium of all fluids (whole plasma, supernatant and albumin fraction) was estimated by a direct microtitration with ethylenediaminetetraacetic acid, Calcein[1] being used as an indicator. The nonprotein-bound calcium was separated by means of a Spinco Model L Preparative Ultracentrifuge maintained at 37° C. during centrifugation. The plasma was centrifuged at 173,000 g for 16 to 20 hours, rotor No. SW 39L being used at a speed of 36,000 rpm. The concentration of plasma protein and the albumin fraction was determined according to the biuret procedure.[24] Citrate and phosphate were measured by methods found to be satisfactory.[3,5] pH was measured by means of a glass electrode and using the Beckman Model G instrument operated at room temperature and corrected to 37° C. In many instances, measurements of ionized calcium were made by the murexide method;[19] but these data are not included, since the results were not entirely consistent with some of the other data regarded as being more reliable. All data are expressed in terms of plasma water unless otherwise stated The correction employed was 99 — 0 75 p where p = Gm. of protein per 100 ml. plasma.

The rats used were adult male animals of the Sprague-Dawley strain maintained on Rockland rat pellets. With one exception, all comparisons on rats were made using controls and experimental animals taken from the same batch. The dogs were female mongrel animals weighing from 16 to 22 Kg. that had been dewormed and maintained for 3 months or more in the laboratory on an adequate diet. Parathyroidectomies performed as described elsewhere[2,15] were considered to be satisfactory if the total plasma calcium value fell to 7 mg. % or less. Blood for analysis was drawn after the animals had been fasting for 12 to 16 hours. All samples for calcium fractionation were collected in tight syringes coated with mineral oil and containing a small amount of heparin. The plasma was maintained under oil until after separation of the various fractions in the ultracentrifuge. Plasma, 4.5 ml., preserved with 50 lambda of chloroform, was used for each plasma separation in the ultracentrifuge. All analyses were carried out in duplicate.

EXPERIMENTAL RESULTS

The ultracentrifuged plasma appeared to have three zones: the upper one (supernatant—Fraction 1) was clear and free of protein; the second, or middle, zone (Fraction 2) was opalescent and contained protein that gave a single peak characteristic of albumin in the analytic ultracentrifuge; and the bottom layer was made up of a gelatinous precipitate.* The calcium of the upper layer was calculated as that not bound to protein (N.P.B.), and the calcium bound to albumin was taken as the difference in calcium concentration between the middle and the upper layers. The calcium and the albumin values always were measured on the same sample of the middle zone.

Effect of Parathyroidectomy on the Plasma Distribution of Calcium of Male Adult Rats (Table 1). A comparison of the distribution of calcium between

* Electrophoretic studies indicate that approximately 80 per cent of the protein in the second layer was albumin.

TABLE 1. THE INFLUENCE OF PARATHYROIDECTOMY ON THE DISTRIBUTION OF CALCIUM IN PLASMA OF ADULT MALE RATS

		No. of Analyses*	Average		Standard Deviation	Probability
pH before centrifuging	Normal†	13	7.40	±	.06	>.1
	Ptx‡	11	7.43	±	.05	
pH after centrifuging	Normal	13	7.41	±	.08	>.1
	Ptx	11	7.45	±	.05	
Total protein Gm.%	Normal	12	6.34	±	.20	>.01
	Ptx	11	6.58	±	.22	< 05
Total Ca mM./L. plasma water§	Normal	12	2.65	±	.06	<.01
	Ptx	11	1.78	±	.26	
NPB Ca mM./L. plasma water	Normal	13	1.57	±	.26	<.01
	Ptx	11	.94	±	.16	
% NPB Ca	Normal	12	59.09	±	2.64	<.01
	Ptx	11	52.46	±	2.34	

* Each analysis equals plasma pooled from 2 rats.
† Average weight for normal rats equals 297 ± 46.5 Gm.
‡ Average weight for parathyroidectomized rats equals 321 ± 57.3 Gm. Ptx = parathyroidectomy.
§ Plasma water is calculated as 99 − 0.75 p where p = Gm. of protein/100 cc. plasma.

the supernatant and the albumin-containing fractions of the plasma of normal and parathyroidectomized rats indicates that a difference does exist. The fraction of the total calcium that was not bound to protein was slightly lower in the parathyroidectomized group. Statistically, this difference from their controls was significant at the 1 per cent level of probability. Although the total binding of calcium by protein did decrease following parathyroidectomy, the decline in the total calcium concentration was relatively greater. A slightly higher value for the total plasma protein concentration was obtained on many of the parathyroidectomized animals as compared with the control group. This difference was small and of questionable significance. No difference could be detected between the moving boundary electrophoretic pattern of the experimental and the control groups

The Influence of Parathyroid Function on the Distribution of Calcium in the Plasma of Dogs. When dogs were parathyroidectomized, the fraction of the total calcium that was not protein bound declined slightly (Fig. 1), as had been observed in the rat. (Curve C). The binding of calcium per *millimol* of albumin* decreased from the normal value, but the decline in calcium concentration was relatively greater, so that the albumin binding of calcium in relation to the total concentration of calcium was relatively greater than before thyroparathyroidectomy. (Curve B).

Administration of parathyroid extract for three successive days produced a hypercalcemia, which was most pronounced on the 4th day and declined somewhat on the 5th day (Fig. 1). The binding of calcium by albumin paralleled closely the total concentration of calcium (Curves A & D). The binding of calcium by albumin goes from approximately 1 mM. of Ca./mM. of albumin in the normal animal to slightly less in parathyroid insufficiency and to 2 mM. of Ca./mM of albumin at the height of the hypercalcemia (Fig. 1, Curve A). The in-

* Average molecular weight assumed to be 69,000.

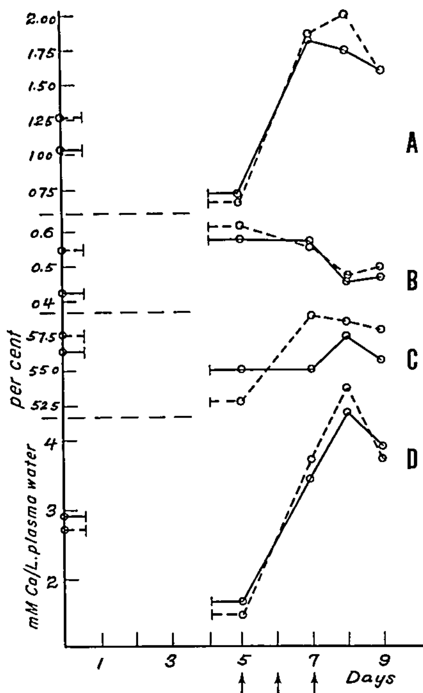


FIG. 1. Plasma calcium distribution of 2 dogs in normal, hypoparathyroid and hyperparathyroid states. (Solid line) Dog No. 34. (Broken line) Dog No. 47.

Ordinate·

Curve A—mM. Ca bound per mM. albumin

Curve B—$\frac{\text{mM. Ca bound per mM. albumin}}{\text{Total calcium in mM.}}$

Curve C—Per cent nonprotein-bound (NPB) calcium of total

Curve D—Total plasma Ca in mM./L.

Abscissae

Same for Curves A, B, C and D

Day O—Normal plasma values prior to thyroparathyroidectomy.

Day 5—Plasma values on 3rd day following parathyroidectomy before hormone administration.

Days 5, 6 and 7—25 u./Kg. of parathyroid hormone.

TABLE 2. PLASMA CALCIUM DISTRIBUTIONS IN THYROPARATHYROIDECTOMIZED DOGS AND FOLLOWING INJECTIONS OF PARATHYROID EXTRACT*

State	Dog No.	Total Protein† Gm./100 cc.	Total Calcium mM./L.	NPB‡ Calcium mM./L.	% NPB Calcium	Calcium Bound to Albumin mM./L.	Albumin mM./L.	mM. Calcium Bound/ mM. Albumin	mM. Ca Bound/mM. Albumin / Total Ca mM
Hypoparathyroid..	34	7.17	1.664	.918	55.17	.542	.562	.964	.579
Hypoparathyroid..	47	6.72	1.462	.768	52.56	.447	.496	.901	.616
Hypoparathyroid..	3	8.42	1.357	.651	47.98				
Hypoparathyroid..	3	7.42	1.307	.544	41.60				
Hypoparathyroid..	25	8.00	1.592	.883	55.48				
Hypoparathyroid .	25	7.25	1.662	.933	56.16				
Mean±S.D.		7.50±.61	1.51±.16	.783±.158	51.49§±.57				
Hyperparathyroid..	34	7.50	4.391	2.520	57.39	1.168	.586	1.993	.454
Hyperparathyroid..	47	8.20	4.736	2.769	58.48	1.278	.570	2.242	.473
Hyperparathyroid..	3	10.69	4.436	2.595	58 49				
Hyperparathyroid..	3	8.22	4.576	2.520	55.07				
Hyperparathyroid..	25	9 63	4.541	2.645	58.24				
Hyperparathyroid..	25	7.52	3.860	2.370	61.41				
Mean±S.D.		8 63±1.27	4.42±.10	2.57±.135	58.18§±.20				

* 25 units/Kg /day for 3 days in succession Value for hyperparathyroid state obtained on 4th day.

† All data are expressed in terms of plasma water unless otherwise stated. Plasma water is calculated as 99 − 0 75 p where p = Gm of protein/100 cc. plasma

‡ NPB = nonprotein-bound calcium of plasma

§ The difference between the 2 groups is significant at the 1 per cent level.

verse relation between calcium binding per mM. of albumin and calcium binding/mM. of albumin in relation to the total calcium is demonstrated by the data presented in Figure 1, Curves A and B. The fraction of total calcium that was N.P.B. was slightly greater during the experimental hyperparathyroidism than when the animals were in a parathyroprivic state (Curve *C*). The data on both animals are consistent in this respect.

Some further data on the binding of calcium by protein in relation to parathyroid function are shown in Table 2. It is evident that the fraction of the total calcium that is N.P.B. was lower in parathyroid insuffi-

ciency than when these animals were made hypercalcemic by injections of parathyroid extract. The differences are more striking for Dog No. 3 than for the other animals, but a change in the same direction was demonstrated in every instance, and comparison of the two groups indicates statistically that the differences are significant.

DISCUSSION

Separation of the N.P.B. calcium by means of the ultracentrifuge eliminates any influence of membrane permeability on the distribution of ions. The manipulations are simple, and the maintenance of a constant pH presents no difficulty and eliminates the necessity of adjusting the CO_2 tension. Duplicate determinations of N.P.B. calcium by this method agree to the limits of accuracy of the method of estimating calcium (1-2%), provided that the pH remains constant.

It is assumed in this study that the albumin-containing zone of the centrifuged plasma contains no sedimentable calcium other than that combined with protein. The studies by Ludewig, Chanutin and Masket[10] appear to have established the validity of this assumption. These authors demonstrated the reproducibility of estimation of the N P.B. calcium by means of the ultracentrifuge, they also showed that a linear relation existed between calcium distribution and protein over a limited concentration of these substances. The in vitro studies by Katz and Klotz, employing differential dialysis,[9] have established a linear relation between calcium ion concentration and the binding of calcium by purified serum albumin over a considerable range of calcium concentration Treatment of the data reported in the present study, according to the expression used by Katz and Klotz, gives a curve for the binding of calcium by albumin with a slope of approximately 2 (Fig. 2). Although more data are needed, and some of the present values do not fall on the curve, it seems evident that the over-all in vivo relation of calcium binding by albumin is similar to that obtained by Katz and Klotz in vitro. However, when one compares the calcium binding by albumin in relation to the total calcium for the two dogs, upon which complete studies were made in the hypoparathyroid and the hyperparathyroid states, both show a decrease in this *ratio* following injections of parathyroid extract (Table 2). These differences are consistent with the change in the percentage of N.P.B. calcium obtained on additional hypopara-

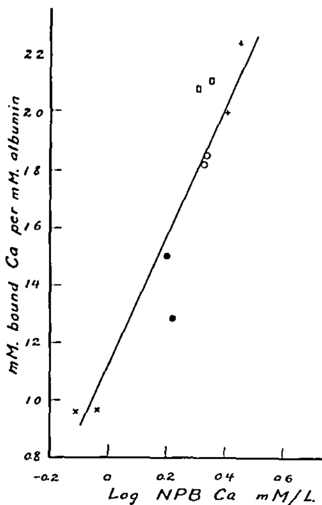


FIG. 2. Relationship between calcium bound to albumin and the N.P.B. calcium of dog plasma in normal, hypoparathyroid and hyperparathyroid states.

● Normal
× Hypoparathyroid state
□ 3rd day of parathyroid extract injection
+ 4th day of hyperparathyroidism
○ 5th day of hyperparathyroidism.

thyroid and hyperparathyroid animals (Table 2) in which no direct measurement of calcium bound to albumin was made.

A change in the calcium binding by albumin may be due to a difference in the affinity of the reactive sites for calcium, which, in turn, is dependent on the calcium ion concentration (Klotz concept). A second possibility is an effect of the hormone on the calcium-binding capacity of plasma protein. The values for pH, citrate and inorganic phosphate determined on the samples of plasma were relatively constant and indicate that the N.P.B. calcium was largely ionized in every instance. This impression was confirmed by analysis in some instances by the direct estimation of ionized calcium.

The older literature contains numerous inconclusive clinical and experimental observations on the distribution of the serum calcium in hypoparathyroid and hyperparathyroid states.[22] McLean, Barnes and Hastings[11] measured the ionization of calcium in experimental hypoparathyroidism and hyperparathyroidism, and found no consistent change in the ratio between the total and ionizable fraction in either state. These authors concluded that the mass law relationship describing the ionization of calcium in plasma held over the entire range from hypocalcemia following parathyroidectomy to the maximal hypercalcemia following the administration of parathyroid extract. From this mass law relationship, one would expect that all hypercalcemic states would be accompanied by an increase in the total diffusible calcium and that the reverse would be true in hypocalcemia, provided that the percentage of the total calcium bound to protein remained constant.

McLean and Hastings[13] emphasized that the significance of the total calcium value could only be assessed accurately when related to the plasma protein concentration. The necessity of directly measuring the ultrafilterable calcium as a means of assessing the calcium distribution was emphasized recently by Prasad and Flink,[16] who found an increase in the ultrafilterable calcium in a variety of clinical conditions associated with hypercalcemia, including hyperparathyroidism. A clinical study of ionized plasma calcium reported by Fanconi and Rose[4] includes the report of a patient with a parathyroid adenoma who had a high value for ionized calcium but relatively slight elevation of the calcium bound to protein. Based on this finding and data on other subjects, they concluded that the excess of parathyroid hormone "had caused a fall in the calcium binding power of proteins." Calculation of the protein-bound calcium in relation to total calcium in two of their hyperparathyroid subjects (Cases 8 & 9), before and after successful surgery, shows a similar trend in relation to parathyroid function to that found in the present data. An earlier study of the ultrafilterable calcium of plasma before and after removal of parathyroid adenomas, reported by Hopkins, Connor and Howard,[8a] failed to demonstrate any consistent change in the percentage of ultrafilterable calcium following surgery despite wide changes in total calcium. In vitro studies reported by Martin and Perkins[14] indicated an increased calcium-binding power by purified plasma albumin prepared from patients with hyperparathyroidism.

Further experimentation will be required to determine the significance of the differences in calcium binding by protein in relation to parathyroid function demonstrated in the present study.

SUMMARY

The plasma calcium of rats and dogs was separated into protein-bound and nonprotein-bound fractions by means of high-speed centrifugation. Hypoparathyroid rats showed a significant reduction in the fraction of N.P.B. calcium as compared with normal animals. Hypoparathyroid dogs showed a

similar difference as compared with the calcium distribution after these animals received injections of parathyroid extract. Direct measurement of calcium binding by plasma albumin in two dogs in relation to parathyroid function showed a reduction in the ratio of albumin-bound calcium to total calcium following injections of parathyroid extract.

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Le Influentia Exercite per Alterationes Experimental del Function Parathyroide Super le Distribution de Calcium de Plasma

Summario in Interlingua

Centrifugation a alte velocitate esseva usate in le separation del calcium plasmatic de rattos e canes in le fractiones ligate a proteina e non-ligate a proteina. Rattos hypoparathyroide manifestava un significative reduction del calcium non-ligate a proteina in comparation con rattos normal. Canes hypoparathyroide manifestava un simile differentia in comparation con lor stato post le injection de extracto parathyroide. Le directe mesuration del ligage de calcium per albumina de plasma in 2 canes revelava un reduction del proportion de calcium ligate a proteina a calcium total post injectiones de extracto parathyroide.

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Additional Evidence in Support of McLean's Feedback Mechanism of Parathyroid Action on Bone*

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It has often been said that one of the most constant physiologic phenomena in vertebrates is the calcium level of the blood. With very few exceptions, such as the laying bird, the calcium concentration in vertebrates is found normally to be between 10 and 12 mg. per 100 ml. of plasma, and in individual animals it is considered to be maintained within more narrow limits. The physiologic need for such a consistent level has not been explained adequately. Calcium is necessary for the blood-clotting sequence, and ionic calcium influences the strength of the heart beat, but neither of these phenomena would appear to require maintenance of the calcium concentration within the narrow limits held normally. A reduction of the ionic calcium concentration of body fluids leads to increased muscular irritability, while an increased concentration leads to a dampening of the passage of impulses across the neuromuscular junction. There is little doubt that normal ionic calcium concentrations are optimal for the proper functioning of the neuromuscular junction. However, there is little reason to assume that values as low as 8 or as high as 14 mg./100 ml would handicap the animal seriously in this regard. Calcium is also important for proper cell membrane function, but how readily this is affected by minor changes in ionic calcium is not known. Accurate control of ionic calcium could be extremely important in cell permeability. It has even been suggested that it is this type of change that regulates the secretion of the parathyroid glands.[4]

The most obvious function of calcium in the vertebrate body is related to the structure of bone. It is in this regard that the value of the maintenance of a constant ionic calcium level is sought, and it is in an attempt to give an explanation of this relationship that this chapter is written. Since it is considered to be well established that the over-all function of the hormone of the parathyroids is to maintain normal calcium levels, one must look to the study of the physiologic actions of this hormone for an understanding of the importance of this close control. A further natural extension of such studies would be to determine how the parathyroids, through their control of the ionic calcium level, aid in the proper functioning of bone.

The first proponent of the action of parathyroid hormone on bone as a mechanism for "feeding back" calcium into the extracellular fluids was Franklin C. McLean, to whom this book is dedicated.[13] His conclusions were based on a series of experiments reported earlier by Hastings and Huggins.[5]

* Most of the work reported here was done with the aid of a grant from the Atomic Energy Commission. The authors are indebted also to Mrs F W. Kraintz, Mr. Bernard Soloff and Dr Richard Davis for help in preparing the manuscript.

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In these experiments calcium was removed from blood by passage over lead phosphate and the resulting solution infused into the dog from which it had been taken. In normal animals the blood quickly regained its original calcium concentration. The surprising part of this study was that the blood concentration in parathyroidectomized dogs also returned to its former level, which was a little over half that of the normal condition. From these studies came the idea of a basic serum calcium level of approximately 7 mg.%. It was suggested that the hormone of the parathyroids evidently increased serum calcium above this level by effecting the release of calcium from bone, thereby maintaining the normal level of 10 to 12 mg.%. It is this concept that is to be examined here, in the belief that work from our own laboratory confirms and extends McLean's thesis. It is hoped that, by so doing, the importance of the parathyroids in the normal growth and remodeling of bone can be more fully appreciated.

SOLUBILITY OF CALCIUM AND PHOSPHATE IN PLASMA

The problem of the maximum quantities of ionized calcium and phosphate that can coexist in mammalian plasma has bothered investigators in this field for over 30 years. The task of solving this is made most difficult by the fact that calcium exists in plasma in several forms other than as an ion and that the plasma is capable, under certain pathologic conditions, of containing large quantities (relatively) of a colloidlike calcium phosphate, which can be maintained as a relatively homogeneous suspension by the physical mixing occurring constantly in blood. It is the thesis of this report that, normally, blood is considerably undersaturated with respect to these two ions and that the control of calcium levels by the parathyroids is not related in any way to the capacity of serum per se for these two ions. The following points are presented in support of this thesis:

1. *Variation of Phosphate Leve[ls in] Mammals.* While calcium levels of pl[asma] *are maintained relatively constant,* [phos]phate levels vary by a factor of almost [...] *in different mammals and in one specie[s] vary by 100 per cent under different c[ondi]tions of growth.* For example, plasma l[evels] *for adult man are considered to be no[rmal] between 2 and 3 mg. P per 100 ml plasma; for the growing child this val[ue is] closer to 6. In the dog, from 4 to 5* [mg.] *P/100 ml.* is considered to be normal, w[hile] in the rat this is raised to between 6 an[d ...] *It is most improbable that the physical c[har]acteristics* of plasma among these vari[ous] mammals are sufficiently different to per[mit] the conclusion that the plasma is satura[ted] *with respect to these two ions in all ca[ses].*

2. *Solubility of Secondary Calci[um] Phosphate.* Shear and co-workers[16] w[ere] the first to state that the ion product *$Ca_3(PO_4)_2$* was inadequate in determini[ng] the solubility of calcium phosphate in bo[dy] fluids. They emphasized later that the i[m]portant salt appeared to be $CaHPO_4$, a[nd] not the tertiary form.[18] By adding secon[d]ary calcium phosphate to serum from se[v]eral different mammals, they were able [to] show that, normally, serum was undersatu[]rated with this salt.[17] Although the initi[al] Ca × P products for the various serum sa[m]ples were quite different, the final product[s] were the same. Recently Strates *et al.*[2] working with activity products, added con[]vincing data for the undersaturation o[f] serum with respect to calcium and phosphate. They stated that mammalian serum normally was undersaturated with respect to $CaHPO_4$ in the absence of the solid phase.

Sobel and Hanok,[19] in in vitro calcification studies, employed an inorganic solution containing 10 mg. per 100 ml. of calcium and 5 mg. per 100 ml. of phosphorus as phosphate. If one accepts the value for ionized calcium in plasma as one half of the total, then Sobel's quantities indicate that plasma should be able to contain at least 20 mg. per 100 ml. of calcium and 5 of phos-

phorus. Hopkins, Howard and Eisenberg[4] found that by adding calcium to human serum they could raise the calcium level to *approximately the above value without any* evidence of the production of colloidal calcium phosphate.

Additional evidence from our laboratory supports the conclusion that in the rat the plasma is approximately 80 per cent saturated with respect to calcium and phosphate in contrast with man, in whom the serum normally is closer to 50 per cent saturated.[5] The data on which our conclusions are based are presented in Table 1. The experimental procedure consisted of adding calcium as calcium chloride and/or phosphate as disodium phosphate to rat plasma and centrifuging at 14,000 G. After high-speed centrifugation, the Ca/2 × P product (½ total calcium × phosphorus) was always in the range of 40 to 50 except when the phosphate was extremely high. This was in contrast with the range of 30 to 40 found in untreated rat plasma. In several of the experiments, centrifugation at 800 G (usual laboratory speed) was employed prior to high-speed centrifugation without an *apparent loss of calcium or phosphate from* solution.

3. Ability of Serum to Remain in Supersaturated State with Regard to Calcium and Phosphate. One of the most convincing proofs that parathyroid function is not related directly to the ability of serum to contain these two ions follows from the observation that in many pathologic and experimental states the quantities of calcium and phosphate measurable in serum are far beyond the solubility of any calcium phosphate salt. This is due to the apparent ability of serum to keep a colloidlike calcium phosphate in suspension. An example of this is seen in the extremely high calcium-phosphate ion product found in plasma taken from nephrectomized animals. Thirty hours after nephrectomy, the phosphorus value may rise above 15 mg.%, while calcium remains in the 10 to 12 range. After

TABLE 1. PLASMA VALUES AFTER HIGH-SPEED CENTRIFUGATION (14,000 G)

Group	Calcium Values BHSC	Calcium Values AHSC	Phosphate (as P) Values BHSC	Phosphate (as P) Values AHSC	$\frac{Ca}{2} \times P$
1. Control	11.30 ± .24	11.30 ± .24	6 79 ± .17	6.67 ± .24	37.7
Ca & PO_4	16.52 ± .29	11.40 ± .23	11.50 ± .26	8.99 ± .27	51.2
2. Control	11.05 ± .35	11.13 ± .32	6.39 ± .30	6.38 ± .31	35.5
Ca Added	16.80 ± .30	14.95 ± .35	6.25 ± .35	5.51 ± .24	41.2
3. Control	10.81 ± .12	10.80 ± .14	5.61 ± .13	5.61 ± .11	30 3
PO_4 Added	10 36 ± .11	9.74 ± .09	10.27 ± .09	10.30 ± .18	50.2
4. Control	11.61 ± .15	11.67 ± .09	6.16 ± .29	6.42 ± .25	37.5
Nephx 30 hrs.	11.07 ± .37	8.03 ± 36	17.29 ± .60	14 88 ± .64	59.7

NOTES:
1. All values given in mg./100 ml. with standard error

$$S E = \pm \sqrt{\frac{\frac{\Sigma X^2}{n} - M^2}{n - 1}}$$

2. Ion product [illegible] of total calcium times total phosphorus.
3. [illegible]
4. Nephx = nephrectomized.
5. Groups 1 to 3 consisted of 8 or more analyses; Group 4, of 4 analyses.

high-speed centrifugation, however, the solubility product falls (Table 1), though not to the expected level.

CALCIUM EQUILIBRIUM BETWEEN BONE AND BODY FLUIDS

Since, under the conditions discussed above, the serum must be considered merely as a reservoir for calcium and phosphate deposited into it, one must look elsewhere to determine the mechanism for the control of calcium levels. The part that the kidney plays in this control, while important, is probably in the form of an adjunct to the more basic phenomenon of the relationship of fluid calcium to the solid phases of bone. This kidney function should be regarded as a safety check to prevent a calcium and phosphate concentration that might lead to calcium deposits in soft tissue, particularly in the kidney itself. Discussions of the role of the kidney can be found elsewhere.[1,21]

Bone usually is considered to consist primarily of tertiary calcium phosphate, and numerous studies have been reported relative to the solubility of this salt as compared with that of prepared bone salts. In all cases the solubility of these salts is far too low to attribute the calcium and the phosphate levels of body fluids to any sort of physicochemical equilibrium between the actual crystal of bone and the fluid itself. This can be demonstrated easily by shaking a few milligrams (1 Gm./100 ml. of plasma) of tertiary calcium phosphate in a beaker with plasma. After centrifugation (14,000 G) both calcium and phosphate plasma values will be found to be reduced to a bare minimum (see Table 3).

McLean's hypothesis gives two levels of calcium equilibration between bone and fluid, both of which are above the solubility of tertiary calcium phosphate, and both, according to our preceding discussion, below the solubility for secondary calcium phosphate. It is the purpose of this section to give evidence in substantiation of the existence of these two levels of bone-serum equilibration for calcium and to attempt to show their relationships to the solubility of secondary and tertiary calcium phosphate.

1. **Existence of a Basic Level of Bone-Fluid Equilibration for Calcium.** The technic of peritoneal lavage has been used for some time in our laboratory to demonstrate the ability of bone continuously to supply calcium to the extracellular fluid compartments in both control and parathyroidectomized rats.[21,24] This has been done variously by the use of the Kolff and Page peritoneal plug[9] or an inguinal glass catheter.[25] A typical group of experiments is summarized in Table 2 (taken from Ref. 12). In all lavage studies, both continuously for as long as 36 hours or discontinuously over a 4-day period, constant calcium levels were maintained in the wash removed from control, nephrectomized or parathyroidec-

TABLE 2. HOURLY CALCIUM LEVELS OF THE RINSE REMOVED FROM PARATHYROIDECTOMIZED AND CONTROL RATS UNDERGOING CONTINUOUS PERITONEAL LAVAGE

Condition		Hourly equilibrium periods							
		1	2	3	4	5	6	7	8
Control	Ca	5.0 ± .16	4.9 ± .23	4.7 ± .13	4.8 ± .24	5.0 ± .13	5.2 ± .16	5.1 ± .20	5.4 ± .03
PTX[2]	Ca	4.9 ± .11	3.8 ± .3	2.9 ± .1	3.0 ± .1	3.1 ± .02	3.3 ± .1	3.4 ± .14	3.6 —

NOTES
1 All values are given in mg./100 ml. with standard error.
2 PTX = animals parathyroidectomized during the first half hour of Period 2
3 Each group consisted of 5 animals.
(Talmage, Elliott & Enders Endocrinology 61:258)

tomized rats. Parathyroidectomy invariably caused a drop in the ability of bone to provide the fluid spaces with calcium. However, the lower level, once reached, was maintained throughout the experimental periods. In rats with intact parathyroid glands, the equilibration value for lavage calcium was found to be approximately 7.0 mg./100 ml, while in parathyroidectomized rats the equilibration value was approximately 4.0 mg./100 ml.[12] The calcium removed was primarily the diffusible fraction, though traces of protein always were found in the peritoneal wash. These experiments suggest that the basic equilibration level between bone and blood is at a calcium level of between 7 and 8 mg./100 ml. of serum. This is in fair agreement with the level proposed by McLean[13] and that determined for the parathyroidectomized dog by Copp[7] in experiments utilizing continuous venous administration of Versene. In the presence of active parathyroid glands the calcium concentration is raised to that found normally in serum.

The relationship of phosphate concentrations to this basic equilibration of calcium between bone and fluid is not clear. Phosphate quantities were always at such levels as to put the product of these two ions far above any possible solubility of tertiary calcium phosphate but still below that of secondary calcium phosphate. Therefore, it is not surprising that the calcium equilibration level does not appear to be affected by small but statistically significant differences in phosphate levels. The factors determining this basic equilibration level are still obscure. The extreme insolubility of tertiary calcium phosphate and the variable phosphate level in the presence of a relatively stable calcium level make it obvious that other factors, such as the effect of the association of bone salts and matrix, or the possible presence of chelating agents, must be involved if this is to be considered a physicochemical relationship.

2. **Results of the Incubation of Intact Bone With Plasma In Vitro.** Numerous studies have been reported in the literature concerning the solubility of bone salts.[7,10,14] However, very few attempts have been made to determine the effect of intact bone on the calcium content of plasma used as incubating medium. The primary problem in this type of study is the maintenance of a constant pH because of the reduction of the buffering capacity of plasma due to the loss of CO_2 and the production of acids by bone during the incubation period. (For the effect of pH on the solubility of apatite crystal, see Ref. 19). In our studies pH was maintained by use of a mixed CO_2 and O_2 atmosphere over the incubation vessel.[27] The time of incubation was limited to 6 hours to prevent a marked effect from the

TABLE 3. INCUBATION OF BONE IN VITRO

Group	Calcium Values	Phosphate Values	pH of Plasma After Incubation
1 Control	10.79 ± 07	6 32 ± .33	
Bone Added	6.13 ± .12	5.99 ± .13	7.30–7.40
2 Control	2.37 ± .10	3.20 ± .10	
Bone Added	5.36 ± .13	6 00 ± .06	7.25–7.40

NOTES

1 Statistical analysis (see Table 1, Note 1)
2 Control group consisted of 7 analyses, the experimental group, of 14 analyses
3. The calcium and phosphate level of Group 2 was reduced by adding $Ca_3(PO_4)_2$ (1 Gm./100 ml. of plasma shaken for 4 hours) and subjecting the contents to high-speed centrifugation.
4 All incubations were for 6 hours.

accumulation of acids. Our results are summarized in Table 3. While an accurate equilibration level for bone and plasma in this type of relationship has not yet been determined, the results indicate that, when plasma containing normal levels of calcium is incubated with bone (rat femur), the calcium level of the plasma falls gradually. In contrast, if bone is incubated in plasma from which most of the calcium and phosphate has been removed previously by shaking with tertiary calcium phosphate, both of these two ions are added to the plasma from bone.

THE SUPERSATURATED STATE OF SERUM WITH RESPECT TO BONE

It has been the contention of the above discussion that there is a basic equilibration between bone and the fluid compartments of the body at the level of between 3.5 and 4 mg. of ionized calcium per 100 ml. of body fluids, or between 7 and 8 mg. of total calcium per 100 ml. of serum. Evidence has been given to show that if the fluid compartments of the body drop below this level, calcium is supplied immediately by bone. On the other hand, if the concentration goes above this level, calcium and phosphate are deposited immediately onto the bone. Since the body fluids are normally above this level, it must be assumed that they are normally in a supersaturated state with respect to the solid phases of bone and that, whenever body fluids are allowed to come in contact with bone, they tend immediately to equilibrate by depositing calcium and phosphate on the surface of bone. Under normal conditions this equilibration level never is reached in the body fluids, due to the continuous action of the hormone of the parathyroids in "feeding back" calcium into the serum from bone against this concentration gradient. Whether or not one assumes the basic level to be a psysicochemical equilibrium, it follows that, for the process of raising serum calcium above this basic level, active removal of calcium is called for. This is supplied by metabolic work initiated by parathyroid hormone on bone structure directly or, more likely, through its action on bone cells.

It must be made clear that this concept for the undersaturation of serum for calcium and phosphate with respect to itself, while at the same time being supersaturated with respect to the solid phases of bone, is not new. The chief recent proponent of this has been Neuman and Neuman,[14] and it has been included in reviews by McLean.[12] Our primary contribution to this concept has been the demonstration of the rapidity with which bone was able to furnish the fluid compartments with calcium up to the basic or supersaturated equilibrium levels, and the rapidity with which the higher level dropped following parathyroidectomy. Such a situation would demand a constant output of parathyroid hormone to counteract the continuous attempt of bone and fluid compartments to equilibrate at the lower level.

The most logical control of parathyroid secretion in such a scheme would be the calcium level (presumably ionic) of the extracellular fluid compartments. While this assumption is generally accepted (though a few investigators adhere to the concept of stimulation of the gland by raised phosphate levels), it has not been proven The problem of control of parathyroid secretion is not germane to the purposes of this report and will be discussed elsewhere.[22]

SITES OF REMOVAL OF CALCIUM FROM BONE

It has been our thesis up to this point that bone is capable of supplying the fluid compartments with calcium by two processes, one of which is a basic, possibly physicochemical, equilibrium upon which is superimposed the "feedback mechanism" of the parathyroids. The purpose of this final section is to demonstrate that these two processes are different physiologically and, for the most part, are concentrated in different areas of bone itself. These conclusions are

made possible by the study of removal of radioactive calcium or phosphorus from bone under various conditions.[21,26]

1. **Distribution of Radiocalcium and Radiophosphorus in Bone.** When either of these radiochemicals is injected into an animal, it is found to localize chiefly in the subepiphysial and periosteal regions of bone. Only over a period of time does it penetrate to the deeper portions of bone. The radioactivity gradually becomes distributed more diffusely by dilution with the stable elements in bone, and eventually some will reach all portions of the bone, though a homogeneous distribution never is achieved.[2] The early deposition is considered by most investigators to be primarily chemical exchange of radioatoms from the fluid with those stable atoms of bone with which the fluid is in physiologic contact. While this can occur with no net transfer of ions, it is in these same areas of growing bone that most deposition of new bone takes place. Under the conditions of the basic equilibration between fluid and the solid phases of bone discussed earlier, it would also be here that the removal of calcium and phosphate should occur, in the event that the fluid concentration fell too low. The following series of experiments from our laboratory are reviewed in order to show that these areas of bone can add calcium to the fluid as well as remove it, depending on the concentration gradient. They also indicate that the parathyroids act primarily to remove calcium from older, more established areas of bone.

2. **Effect of Time on the Removal of Radioisotopes from Bone.** The basic technic in these experiments was that of continuous peritoneal lavage with calcium-

TABLE 4. EFFECT OF PARATHYROIDECTOMY ON REMOVAL OF CALCIUM AND PHOSPHATE BY CONTINUOUS PERITONEAL LAVAGE

	Exp. Cond.	No. of Animals	Lavage Periods (60-min. washes) 2	4	6	8	10	12	Total for 12 hrs.	Diff.
Ca mg./hr. ..	Nephx	11	1.66	1.52	1 63	1.76	1.68	1.85	20.0	
	PTX-Nephx	11	1.06	1.06	1.20	1.30	1.34	1.29	15.0	−25%
Sr^{85} (c/m) (18 hrs.) ..	Nephx	5	94	95	102	112	107	103	1220	
	PTX-Nephx	5	101	101	108	105	100	92	1210	
Sr^{85} (c/m) (3 wks.) ..	Nephx	6	90	90	100	107	103	112	1210	
	PTX-Nephx	6	66	70	73	74	76	92	890	−27%
PO_4 mg.P/hr.	Nephx	11	2.4	2 0	2.1	2.3	2.2	2.2	27.0	
	PTX-Nephx	11	2.1	1 8	1.9	2.0	2.0	2.0	24.0	−11%
P^{32} (c/m) (18 hrs.) ..	Nephx	5	120	103	97	103	86	71	1160	
	PTX-Nephx	5	110	98	103	95	92	66	1130	
P^{32} (c/m) (3 wks.) ..	Nephx	6	114	91	90	102	93	104	1190	
	PTX-Nephx	6	90	75	77	85	82	83	980	−17%

NOTES:

1. Data in this table summarized from data reported in *Endocrinology*, vol. 65, p. 1, 1959. For statistical evaluation and use of Sr^{85}, see this reference.
2. Nephx = nephrectomized only; PTX-Nephx = parathyroidectomized and nephrectomized
3. Sr^{85} or P^{32} (18 hrs) = radioactivity injected 18 hours before lavage.
4. Sr^{85} or P^{32} (3 wks.) = radioactivity injected 3 weeks before lavage.

and phosphate-free fluid, performed in nephrectomized animals with and without parathyroids.[26] As discussed above, this technic will produce a relatively constant removal of calcium from bone in both parathyroidectomized and parathyroid-intact animals, the amounts of calcium removed in the former group remaining at about two thirds the level of the controls. In addition, all animals were given radiocalcium or radiophosphorus (or Sr^{85} and P^{32} simultaneously) either in a single dose 18 to 24 hours prior to the start of the lavage or in 5 daily doses starting 3 weeks before the lavage was run. The results of such experiments are summarized in Table 4.

During the first 24 hours after injection of a radioisotope, its localization in bone is, as described above, in definite areas. That these are the areas in which most of the basic equilibration between bone and fluid occurs is indicated by the fact that the removal of recently injected radioactivity is not affected by parathyroidectomy but remains constant in both groups of animals. This is in contrast with the drop in rate of calcium and phosphate removal that follows parathyroidectomy. On the other hand, if the radioactivity is allowed to remain in the bones from 2 to 3 weeks, thereby permitting some penetration of the deeper areas of bone, the removal rate of both the stable forms of calcium and phosphate and their radioisotopes drops after parathyroidectomy. This suggests that while the basic level of equilibration between bone and fluid occurs in those areas of closer physiologic contact, the parathyroids call upon established stores of bone to feed calcium into the fluid, thereby maintaining the normal calcium levels

The studies reviewed above[26] indicate further that the calcium removed by the parathyroids is provided by the dissolution of established bone crystals. In these stu[illegible] the drop in the rate of removal of calc[illegible] phosphate and the radioisotopes of [illegible] elements after parathyroidectomy was lowed. In the lavage experiments summarized in Table 4 it can be noted that parathyroidectomy reduced the stable calcium to phosphorus ratio in the wash by a factor of 2:1 while lowering simultaneously the ratio of the radioactive counterparts by a 3:2 ratio. While the actual chemical formula for apatite crystal is not agreed upon, the ratios of 2:1 by weight and 3:2 with respect to the number of atoms of each usually are accepted. Since the radioactivity is affected only if it has been in the bone for long periods of time, the conclusion is drawn that the calcium supplied the fluid by action of the parathyroids is obtained from the dissolution of bone crystals in areas of bone in which the crystals were not readily available for equilibration with the fluid compartments.

Another indication of the relatively specific areas of bone on which the parathyroid hormone may call for needed calcium is the relationship of hormone activity to osteoclastic activity. Heller *et al*[6] have shown clearly that parathyroid extract administration increased the numbers of osteoclasts present in long bone. The results of work in progress in our laboratory indicate a semiquantitative increase in numbers of osteoclasts in subepiphysial and trabecular areas of long bones of rats following increased endogenous activity of the parathyroid glands. This is not considered to be proof of the direct action of the hormone through these cells; nevertheless, it is regarded as further evidence that there are specific areas of bone from which the hormone is able to procure the calcium needed to raise serum concentrations above the basic equilibrium level.

SUMMARY

It has been the thesis here that there is a [illegible] physicochem[illegible] [illegible]brium between [illegible] and the [illegible] body, that this [illegible] place [illegible] [illegible] areas of bone [illegible] able to [illegible] [illegible]apidly with the [illegible] [illegible]ids; and [illegible] of this equi-

librium process, these areas of bone are capable of either supplying the fluid compartments with calcium and phosphate or removing these ions from fluid by depositing them in bone. However, because of the condition of supersaturation of the body fluids with respect to the solid phases of bone, the process of equilibration at this basic level is normally a one-way gradient from fluid to bone.

The reason for the supersaturation of these fluids in respect to bone is primarily due to the feedback mechanism of the parathyroids. The hormone dissolves bone crystals and discharges the calcium and the phosphate into the body fluids against the concentration gradient, thereby maintaining the standard 10 mg./100 ml. serum level for this ion. Therefore, the action of the parathyroids in this regard must be continuous, for, without the hormone, the basic equilibrium level is reached in a very short time. If, as is generally assumed, the secretion of the parathyroids is controlled by the ionic calcium level of the circulating fluids, then the sensitivity of the gland itself to variations in ionic calcium content of the fluid passing through it would account for the constancy of the calcium levels found in the blood of most vertebrates.

Whether or not a well-controlled and constant calcium level is necessary for proper bone metabolism and growth might be questioned, but it certainly has two definite advantages. The first of these is that such a situation provides, by the condition of supersaturation, a constant rate of deposition of bone salts into the pertinent areas of growing bone. The second is that, since the exogenous supply of calcium is rarely sufficient to satisfy the conditions of this deposition while fulfilling the high requirement for serum calcium, the parathyroids are called upon continuously to put calcium back into the circulation. Such a situation aids the normal process of bone remodeling that is so important, particularly to the growing animal.

A final point to be made is that, while the calcium level is constant, that for fluid phosphate is not. In such a supersaturated state, to change the phosphate level in the presence of a constant calcium level would tend to alter the rate of deposition of these salts in bone. Therefore, the mammalian body has a mechanism for making certain that bone, at all times, has the salts that it needs for growth: in conditions where growth rate is elevated, the physiologic activities allow for an increased rate of precipitation due to the higher phosphate levels found in the body fluids of growing animals. However, all this is accomplished in a fluid medium that, when separated from bone, is not in itself saturated with these two ions necessary for bone growth and remodeling.

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SUMMARY

It has been the thesis here that there is a basic physicochemical equilibrium between bone and the fluids of the body; that this takes place primarily in those areas of bone that are able to equilibrate rapidly with the body fluids; and that, because of this equi-

librium process, these areas of bone are capable of either supplying the fluid compartments with calcium and phosphate or removing these ions from fluid by depositing them in bone. However, because of the condition of supersaturation of the body fluids with respect to the solid phases of bone, the process of equilibration at this basic level is normally a one-way gradient from fluid to bone.

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Observationes Additional in Supporto del Conception de McLean de Un Mechanismo de Feedback in le Effecto del Parathyroide Super le Osso

Summario in Interlingua

Iste reporto es redigite in supporto del these que il existe duo nivellos de equilibration inter osso e sero con respecto al concentration de calcium. Le processos de equilibration a iste duo nivellos es continuemente active e opponite le unes al alteres. Le nivello plus basse es determinate per un relation physicochimic inter le liquidos del corpore e le areas de osso que se trova in contacto physiologic con le compartimentos de liquido. A iste nivello le transition de calcium es normalmente orientate ab le liquidos del corpore verso le osso, sed isto pote esser revertite experimentalmente per technicas como le lavage peritonee o le administration continue de versena. Equilibration a iste nivello es approchate solmente in animals parathyroidectomisate. Le nivello plus alte es un resultato del continue liberation de calcium ab le osso verso le compartimentos de liquido per un action de hormon parathyroide que effectua le dissolution de crystallos in osso in areas que non se trova in contacto physiologic directe con le liquidos del corpore. Il es iste continue action del hormon parathyroide que mantene le remarca-

bilemente constante nivellos de calcium que es incontrate in le sero de omne mammales.

Le importantia physiologic del mentionate duo nivellos de equilibration es apparentemente a vider in le dominio del crescentia e del refactionage de osso. Le condition de supersaturation del liquidos con calcium permitte le constante precipitation de sales ossee in le pertinente areas de osteogenese. Del altere latere, viste que le provision exogene de calcium suffice rarmente a satisfacer le conditiones del gradiente fundamental de concentration, simultaneemente con satisfacer le alte requirimentos de calcium seral, le parathyroides es continuemente stimulate a retroducer calcium in le circulation. Iste processo adjuta le processo normal del refactionage de osso, lo que es importante—particularmente in le animal que se trova in stato de crescentia.

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Parathyroid Enlargement in Laying Hens on a Calcium-Deficient Diet*

W. BLOOM, A. V. NALBANDOV AND M. A. BLOOM†

Some years ago we noticed that the parathyroid glands hypertrophied in laying hens on a diet inadequate in calcium.[1] More recently, we have studied this hypertrophy in laying hens kept on a carefully controlled full diet and on the same diet low in calcium only. It is these observations that we wish to report here. They are of interest in view of the findings of Crawford *et al.*,[2] who observed that in rats on a low-calcium diet the hypertrophy of the parathyroid glands was due to the absence of vitamin D rather than to calcium deficiency. (See also the review by McLean and Budy.[3]) As will be shown, this conclusion does not hold for our hens, presumably because of the unusual calcium metabolism associated with egg-laying.

The following basal diet fed our hens was recommended by Dr. H. M. Scott as being adequate except for its calcium content:

BASAL DIET

Ground yellow corn	60.2
Soybean meal	20.0
Alfalfa meal	5.0
Dried distillers' solubles (corn)	5 0
Condensed fish solubles	2 5
Iodized salt	0 5
$NaH_2PO_4 \cdot H_2O$	1 0
$MnSO_4$—feed grade	0 25
A and D feeding oil (600 I.U /Gm of D, 3,000 I.U./Gm. of A)	0.15
Riboflavin—100 mg /100 lbs	+
Ca pantothenate—210 mg /100 lbs	+
	94 60

The control hens were fed additional calcium as limestone (Diet A), whereas those in the experimental groups received the basal diet (Diet B).

	DIET A	DIET B
Basal	473	473
Ground corn	—	27
Limestone	27	—
Total	500	500

The calculated analysis of the control and the experimental diets was the same for phosphorus (0.61%). The percentage of calcium was 2.25 in Diet A and 0.17 in Diet B. The vitamin D supplement was the same in both. Since each hen ate about 125 Gm. of this diet per day, she received about 112 I.U. of vitamin D daily.

Two experiments were carried out in which the parathyroid glands were weighed at the time of autopsy. In the first experiment, both glands of each bird were weighed. In the second experiment, one gland only from each hen was weighed, while the other gland was fixed and prepared for histologic study. Experiment 1 consisted of 9 hens maintained on the low-calcium diet for 10 or 11 days and 5 controls on the normal diet. In Experiment 2, 14 hens were fed the same calcium deficient diet for 10, 20 or 30 days. Fifteen hens on the normal diet served as con-

* This work has been aided by grants from the Section on Arthritis and Metabolism of the U. S Public Health Service and the Dr Wallace C. and Clara A Abbott Memorial Fund of the University of Chicago

† [illegible] men [illegible] Department [illegible] Illinois.

TABLE 1. EFFECTS ON LAYING HENS OF LOW-CALCIUM DIET

Hen No.	Days on Test	No. of Eggs Laid	Blood Calcium mg. %	Weight of Both Parathyroid Glands in mg.	Osteoid in Medullary Bone
Low-Calcium Diet					
31	10	2 (1 S*)	16.6	48.2	Present
32	10	5 (1 S)	12.6	15.0	Trace
33	10	5	13.2	50.8	Present
34	10	4 (2 S)	16.8	37.4	Present
35	10	7	12.8	97.2	Present
36	11	7	11.2	77.4	Present
37	11	7	10.8	77.0	Present
39	11	7	9.6	112.4	Present
40	11	2 (1 S)	21.2	45.0	0
Average		5	13.87	62.27 (31.14 = average for 1 gland)	
Normal Calcium Diet					
41	11	Not laying	21.6	36.6	0
42	11	9	20.8	40 4	0
43	11	6	20.8	41.0	0
44	10	9	18.4	16.3	0
45	10	8	18.4	18.3	0
Average		6	20.00	30.52 (15.26 = average for 1 gland)	

* S = soft-shelled egg.

trols here. The laying records of all these hens were kept, and a variety of observations were made, some of which will be reported elsewhere. Samples of bone were taken at autopsy, and the findings on these will be referred to here only briefly.

The results of these experiments are presented in Tables 1 and 2. In each instance the average weight of the parathyroid glands was much greater for the group on the calcium-deficient diet than for its controls, despite wide variations between individual hens within each group. In the experiment of 10 or 11 days' duration (Table 1), the weight of the parathyroids was high in 8 of the 9 hens on the experimental diet. Six of these birds continued to lay throughout the test period. Even those laying sporadically (Nos. 31, 34 & 40) had heavier parathyroids than the average for the control group.

In order to compare the parathyroid weights of Tables 1 and 2, it is necessary to divide the weights of Table 1 by 2, since in Table 2 only one parathyroid was weighed, the other one being removed for histologic study, as mentioned above. When this adjustment has been made, the results of the two experiments are similar. In the first low-calcium group, the average weight of one parathyroid was 31.1 mg., as compared with 38.4 mg. in the second group, whereas the controls were 15.3 mg. and 13.5 mg., respectively.

The size of the parathyroid showed no correlation with the length of time on the low-calcium diet. In the groups maintained on this diet for 20 or 30 days, regular egg-laying ceased for the most part after the first 8 or 10 days; after this there were only a few eggs, and most of them had soft shells Thus, in the 20-day group, of a total of 28 eggs, only 4 were laid after the first 10 days, and 3 of these had soft shells. Similarly, in the 30-day group, of a total of 47 eggs, only

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Age Changes in Human Bone

JENIFER JOWSEY*

Recently microradiography has been used to study the microscopic appearance of bone tissue[2,4,6,10] since it provides information about the distribution of mineral within the tissue that is not obtained in conventional histologic preparations. As this method of investigation becomes more widely used, a need arises for establishing the variations of structure found in both normal and pathologic skeletal tissues. This report is an attempt to describe the microradiographic appearance of normal bone and to relate the variations of structure to the age of the individual. The material is part of a survey of normal bone, including chemical analyses and quantitative microradiography, that is being carried out in this laboratory, and some preliminary results recently were reported.[8]

METHODS

Normal bone material has been collected from 24 individuals in whom death occurred suddenly either as the result of accident or acute illness. A cross section about 0.5 cm. thick is taken from the mid-point of the femoral shaft and divided into four quadrants These are embedded in methyl methacrylate and cut into sections approximately 100μ thick with a circular metal saw.[3] The sections may be ground more accurately to a thickness of 100μ on ground glass sharpened with fine carborundum powder. Contact microradiographs are made by methods similar to those already described.[5,8] The sections are exposed on Kodak maximum resolution plates using a Raymax 60 unit, with a continuously evacuated demountable tube and half-wave rectification. The unit is operated at 20 kv., and a copper target has been chosen since its characteristic radiation is absorbed selectively by hydroxyapatite, the bone mineral. High resolution and evenness of beam are obtained by using a small focal spot, less than 1 mm wide, and a target-to-film distance of 20 cm.

A detailed description or survey is carried out on an area that consists of one third of the entire femur cross section. To obtain comparable samples from each individual, photographs are made of the microradiographs of the quadrants and a sample is

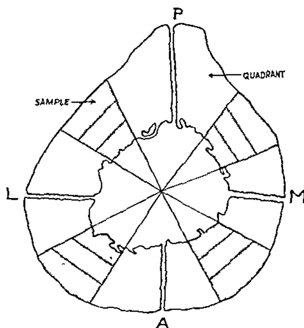


FIG 1 Diagram of femur cross section composed of the 4 quadrants One sample is taken from each quadrant.

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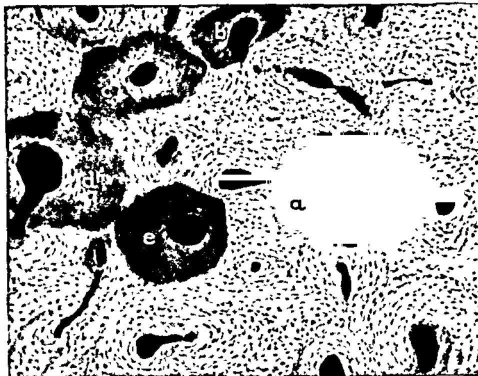


Fig. 2. Microradiograph of the femoral cortex of a 40-year-old male. Bone formation is taking place in Osteone a. The bone is of low mineral density, and the lamellae are arranged concentrically round the central canal. Osteones b, c, d and e are of somewhat higher density and have an inner band of lamellae of increased density, indicating that they are not in the process of active bone formation. (× 110)

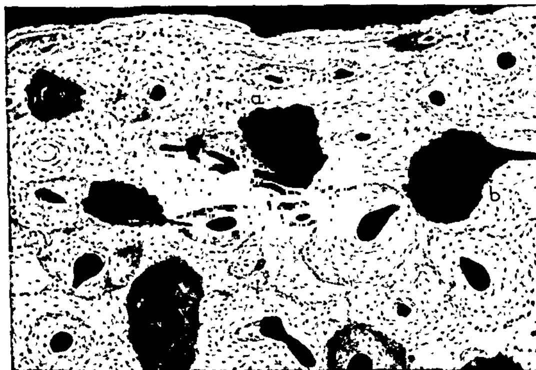


Fig. 3 Microradiograph of the femoral cortex of a 57-year-old male. Resorption is taken place at "a" The walls of the resorption cavity are irregular and of high mineral density, and the lamellae are arranged randomly with respect to the surface of the cavity. Bone formation has just started in Osteone b (× 105)

drawn out on each (Fig 1). Because there appear to be considerable differences between bone on the inner and the outer parts of the cortex, each sample is divided into three simply by dividing the sides of the sample into three equal lengths. This produces a rather arbitrary division into what has been called "endosteal," "middle" and "periosteal" bone. Each sample contains these three in the same proportion as they are present in

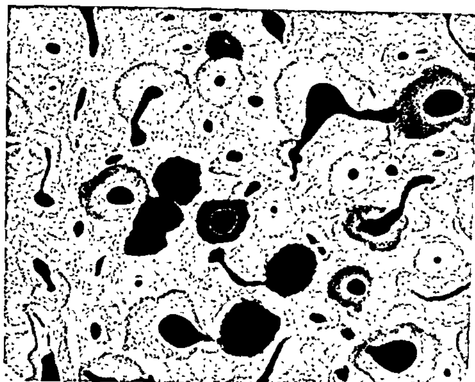


FIG. 4. Microradiograph of the femoral cortex of a 40-year-old male. The osteones are of varying degrees of mineral density, and a number of them are less than three quarters closed. (×47)

the entire cross section. Since no significant differences were found between the four quadrants of each cross section, the results are obtained as averages of the "endosteal," the "middle" and the "periosteal" bone in the four quadrants, and in the histograms these have been averaged to produce a single number for each individual. For the first 13 individuals the remaining two thirds of the sections were also studied to discover if a sample of one third was an adequate representation of the whole section. No great differences were found between the sampled areas and the rest of the section.

The unit of structure of cortical bone is the haversian system or osteone that is formed by the deposition of concentric lamellae within an irregularly cylindric cavity, the fully formed osteone having a central canal carrying blood vessels. Approximately 50 per cent of the area of a cortical bone section consists of these osteones, remaining 50 per cent being bone and the remains of osteones that been replaced partially and no longer p sess a central with a potentiality surface. It that are ble for th cortical bone

and this survey has been limited to a description and semiquantitative analysis of the activities occurring on the surfaces of the osteones, the state of mineralization of the osteones and other features related to their metabolism.

Bone formation and resorption are perhaps two of the utmost important features of bone structure, since they are the means of renewal of the bone tissue. These two activities occur on bone surfaces only; therefore, it has seemed to be most logical to measure the amount of bone surface occupied by either of these two activities.[8] Bone formation is recognized in a microradiograph (Fig. 2) by the presence of lamellae of low mineral density running parallel to the central canal of the osteone and forming a smooth surface. Bone resorption is characterized by an uneven crenated surface of highly mineralized bone, and the may be angle to the (Fig 3). Th the surface by either resorption d and percentage in the proc rapidly to

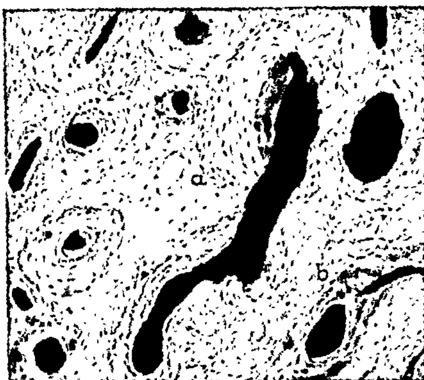


FIG. 5. Microradiograph of the femoral cortex of an 84-year-old male showing a plugged canal at "a" and filled lacunae appearing as white specks at "b." (×140)

a degree of mineralization corresponding to over half that which it will achieve eventually. Further mineralization takes many months, so that in any microradiograph there will be osteones at various degrees of mineralization, as shown in Figure 4. Amprino[1] relates the distribution patterns of mineral densities of osteones to the process of bone formation. He has used the primary periosteal bone as a reference with which to compare the differences in the mineral densities in individuals of different ages, and he found more osteones of low density in a growing child than in an adult. In the present report the number of osteones of particularly low density has been estimated as an indication of recently formed bone. Quantitative microradiography carried out in this laboratory[9] on the same areas of bone indicate that these osteones of particularly low density correspond to a degree of mineralization approximately 75 per cent or less of that achieved by the adjacent interstitial primary bone

Osteones of a somewhat higher mineral density do not appear to be related to new bone formation but to a failure to achieve full mineralization for an unusually long time. Such osteones have also been counted and correspond approximately to between 75 and 85 per cent of the degree of mineralization achieved by interstitial bone. Since the low density osteones are distinguished only visually and their degree of mineralization is based on comparisons with the interstitial bone density in each individual, any quantitative data must be regarded as only approximate. In each sample the total number of osteones is counted (there are from about 1,500-2,000 osteones in the sampled areas in each individual), and the numbers of low density osteones, those below 75 per cent and those between 75 and 85 per cent of complete mineralization, are expressed as percentages of the total number of osteones present.

A feature of bone structure particularly interesting in relation to osteoporosis is the presence of osteones that are incompletely closed; that is, the central canal is large and remains so. Frequently such osteones possess an inner ring of lamellae of slightly higher density than the rest of the osteone, in which respect they resemble most fully formed osteones where the central canal is small. In this survey, osteones have been

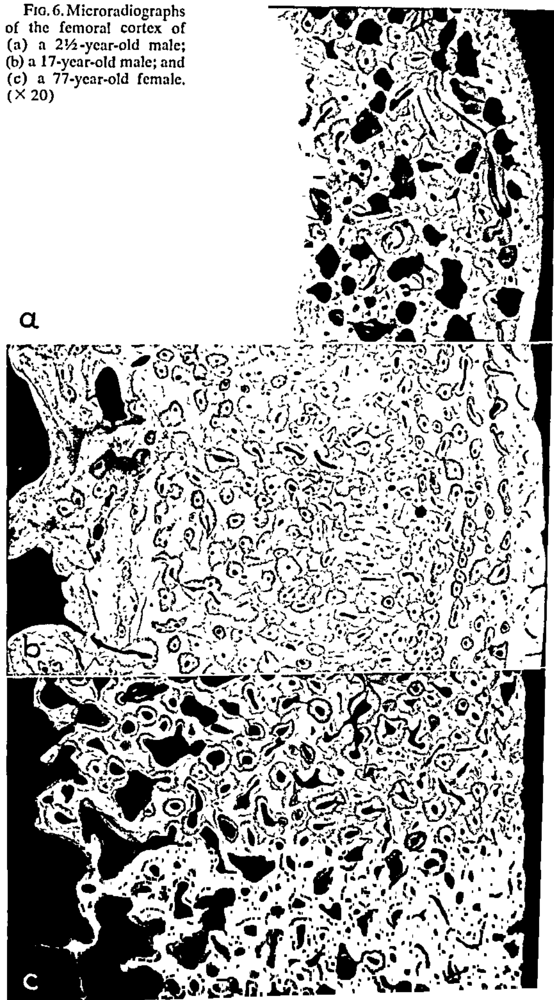


FIG. 6. Microradiographs of the femoral cortex of (a) a 2½-year-old male; (b) a 17-year-old male; and (c) a 77-year-old female. (× 20)

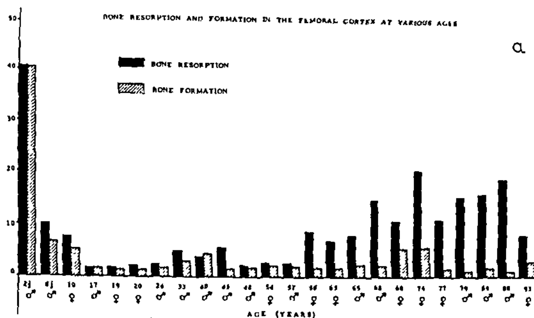
counted as incompletely closed if the diameter of the central canal is a quarter or more than a quarter of the cement-line diameter of the osteone; that is, if they are less than three quarters closed. This number has been expressed as a percentage of the total number of osteones present in the sample; it represents the porosity of the bone and will include most forming osteones and all resorption cavities.

Occasionally the central canal of an osteone becomes plugged with calcified connective tissue that is of a higher mineral density than the surrounding bone, as seen in Figure 5. Similarly, osteocyte lacunae may become filled with bone mineral appearing as white specks in the microradiographs (Fig. 5). Both these features—the plugged canals and the filled lacunae—have been described by Rowland,[6] analyzed quantitatively and related to radium burden in radium-burdened humans. Though these two features appear only infrequently in normal individuals, it is important to establish their occurrence and to discover if their incidence varies with age.

RESULTS

From the microradiographs themselves (Fig. 6) as well as the histograms (Fig 7) it is evident that there are considerable variations of structure with age. Young individuals show a high percentage of both bone formation and resorption, indicating a high turnover rate (Fig 6, a); the high value for porosity of the bone (Fig. 7, c) is the result of a large number of forming osteones and resorption cavities. In young adults there is apparently a remarkably low turnover rate: there is little bone formation and destruction (Fig. 7, a); consequently, few systems of less than 75 per cent of full mineralization. Most osteones close fully and attain a high mineral density fairly rapidly, so that the bone has a compact appearance and there is little variation in density (Fig. 6, b). Later in life there is a gradual increase in the amount of resorption, particularly in the "endosteal" bone, which in individuals over 70 years of age may have up to 25 per cent of its surface occupied by resorption. There is also evidence[6] that it is taking place rapidly, since many of the surfaces of the resorption cavities have hollowed-out extensions typical of fast resorption. There is little evidence of an increase in either bone formation or the number of osteones of less than 75 per cent of complete mineralization. From about 60 years of age onward there is a dramatic increase in the number of osteones that are less than three quarters closed (Fig. 7, c), particularly in the "endosteal" bone. Unlike the young individuals, this increase is due, to a large extent, to osteones that have remained incompletely closed rather than to the presence of a large number of forming osteones and resorption cavities. At this age also there is a marked increase in the number of osteones that are between 75 and 85 per cent of complete mineralization (Fig. 7, b): in some individuals there are only relatively few osteones that approach the degree of mineralization present in the primary interstitial bone. The microradiographic appearance of bone from individuals of these ages is one of porosity and of great variation in mineral density (Fig. 6, c).

Both plugged canals and filled lacunae are found in young adults, but only occasionally, while in old age there may be quite a number of both. Most frequently the plugged canals appear in the "periosteal" bone where, in both the 84- and the 88-year-old individuals, 3.4 and 3.5 per cent, respectively, of the canals are plugged, and the averages of the whole sample are 1.5 and 1.9 per cent, respectively, values that approach the number quoted for an individual with a terminal body burden of 1.2μc. Ra^{226}.[Ref 6] Though older individuals are more likely to show more plugged canals, there may be less than 1 per cent in some old individuals. The same is true of the incidence of filled lacunae; more are present in older people, but there may be some individuals above 70 years of age with an average of



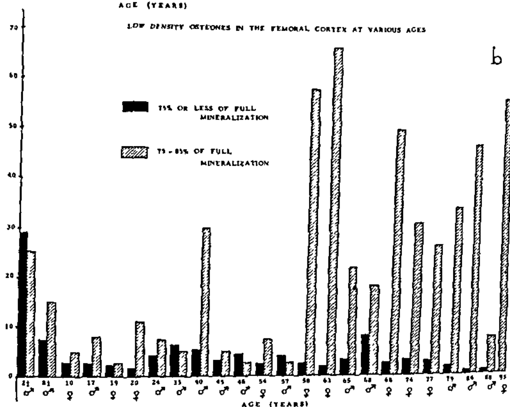
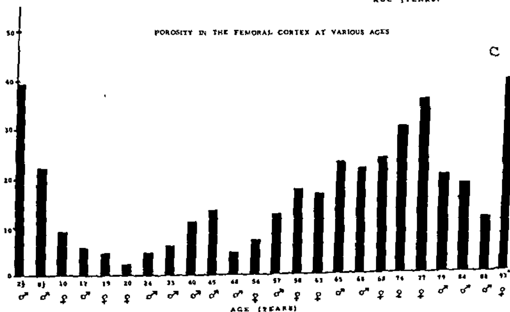


Fig. 7. Age changes in haversian bone of the femoral cortex.



less than 1 per cent. Most often filled lacunae appear in the interstitial bone, where large areas of bone containing over 15 filled lacunae may be found in some individuals.

The microradiographic appearance of hypermineralization must take place some time after the death of the tissue, since a considerable interval must elapse before the tissue and the cell spaces become mineralized. Indeed, canals are found at all stages of plugging, though only completely occluded canals have been counted, and some lacunae appear to be filled with bone mineral to produce a density similar to that of the adjacent bone but not yet manifesting as the typical hypermineralized speck. Therefore, even the low incidences of plugged canals and filled lacunae reported for normal bone must reflect considerable interruptions in vascular supply and a significant amount of cell death in the bone tissue.

CONCLUSIONS

This survey of the structural appearance of normal bone is very much a preliminary study, but it does give some indication of the variations that are found and their relation to the age of the individual.

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Alterationes Occurrente in Osso Human con le Avantiamento del Etate

Summario in Interlingua

Osso normal esseva colligite ab 24 subjectos de etates de inter duo e medie e 93 annos. Le alterationes structural relationate al etate esseva studiate in le mesodiaphyse femoral Methodos microradiographic esseva usate. Le area investigate includeva un tertio del total section femoral transversal. Le investigation esseva restringite al unitate structural de osso cortical que es cognoscite como systema haversian o osteon.

Le metabolismo de osso occurre como resorption e neoformation de osso al superficie del ossos individual. Le proportion del total disponibile superficie que es occupate per resorption o per neoformation de osso esseva mesurate. Esseva etiam determinate le numero del osteones de basse densitate, e le porositate del osso esseva estimate. Duo altere aspectos esseva includite in le studio: Osteones in que le canal central es plenate de materia calcificate e lacunas osteocytic que es plenate de mineral de osso.

Subjectos de basse etate exhibi un alte procentage de neoformation e resorption de osso. Isto reflecte un alte rapiditate del metabolismo. In juvene adultos, le activitate es remarcabilemente inerte. Le osso exhibi un apparentia compacte con pauc variabilitate de densitate. Plus tarde in le curso del vita, il occurre un augmento in le activitate resorptive, sed il ha pauc indicios de un correspondente augmento del neoformation de osso. Il occurre un marcate augmento del porositate a etates avantiate, causate in grande mesura per le presentia de osteones que es incompletemente formate. Il occurre etiam un augmento del numero de osteones que es incompletemente mineralisate e del numero de canales obturate e de lacunas plenate de mineral de osso.

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Epidemiology of Fracture in Aged Persons*

A Preliminary Investigation in Fracture Etiology

GÖRAN C. H. BAUER, M.D.†

INTRODUCTION

Fracture of the neck of the femur occurs predominantly in the aged, requires long periods of bed rest in hospitals or nursing homes and has a high incidence of complications. As the proportion of aged people in the population continues to increase, fracture of the neck of the femur becomes a major problem for orthopaedic surgeons and hospital administrators.

Quantitative evidence of the magnitude of this problem now is available. In the Eastern Region, Scotland, it is anticipated that the rise in the number of cases annually of fracture of the neck of the femur will have risen by 50 per cent in the period 1952 to 1975.[8,9] In the Oxford area in England, the incidence of fracture of the neck of the femur is five times higher in subjects between 70 and 79 years of age than in those between 50 and 59.[3]

As part of experimental and clinical investigations of bone metabolism, an epidemiologic study of fracture has been started in the city of Malmö. The purpose of this study is to evaluate the importance of biologic factors in the occurrence of fracture. In this chapter the author presents preliminary results of surveys of fractures of the neck of the femur and the lower end of the forearm. These results will be used in a discussion of the cause of fracture in the aged.

MATERIAL‡

The present study comprises (a) 956 cases of fracture of the neck of the femur and (b) 1,192 fractures of the lower end of the forearm. Only fresh fractures in subjects over 20 years of age are included. The periods of observation were (a) from 1949 to 1958 and (b) from 1953 to 1957.

These series are completely unselected and are estimated to constitute 95 per cent

‡ A detailed presentation of this material will be published elsewhere in collaboration with Dr. P. A. Alffram.

TABLE 1. ADULT POPULATION OF MALMÖ IN 1955

Age Group	(a) Women	(b) Men	Ratio a/b
20–24	6.456	5.887	1.09
25–29	7.504	7.116	1.05
30–34	8.710	7.993	1.08
35–39	8.745	8 285	1.06
40–44	8.813	8.189	1.06
45–49	8.675	7.693	1.12
50–54	7.402	6.431	1.15
55–59	6.167	5.311	1.16
60–64	5.293	4.165	1.27
65–69	4.569	3.490	1.30
70–74	3.633	2.782	1.30
75–79	2.504	1.728	1.40
>80	1.732	1.170	1.48

* Presented in part at a joint staff meeting at the University Clinics, Malmo, Sweden, held on December 15, 1958

Financial support of this work was obtained from Josiah Macy, Jr. Foundation, New York, N. Y., and the [illegible]

Al [illegible]

TABLE 2. FRACTURE OF NECK OF FEMUR IN MALMÖ 1949-1958

Health Prior to Fracture	Men: Degree of Trauma, Slight	Men: Degree of Trauma, Severe	Men: Total	Women: Degree of Trauma, Slight	Women: Degree of Trauma, Severe	Women: Total
Good	45 (3)	81 (2)	126 (5)	355 (32)	59 (4)	414 (36)
Poor	59 (15)	22 (1)	81 (16)	271 (50)	42 (4)	313 (54)
Unknown	2 (0)	2 (0)	4 (0)	16 (2)	2 (0)	18 (2)
Total	*106 (18)*	*105 (3)*	*211 (21)*	*642 (84)*	*103 (8)*	*745 (92)*

The figures in parentheses represent the number of deaths within 3 months of fracture

of such fractures diagnosed in the population during the period under study. The material has been divided into two groups, according to degree of trauma. *Moderate trauma* means less than or equivalent to a fall on the ground level; *severe trauma* includes all other falls and all traffic accidents (including falls from bicycles).

In 1955 the city of Malmö had a population of 209,473. The age and the sex distribution of adults in this population is shown in Table 1.

RESULTS

FRACTURE OF NECK OF FEMUR

Fracture of the neck of the femur is defined here as comprising all fractures of the femur proximal to and including the *minor trochanter, excepting those that are* predominantly subtrochanteric.

The entire material (Table 2) consists of 745 women and 211 men, a ratio of women to men of 3.5 to 1, which exceeds that of any single age group in the population (*Table 1*).

In men the two types of trauma were equally common, while in women moderate trauma was more than six times as frequent as severe trauma. The over-all mortality (within 3 months of fracture) was 12 per cent. The mortality was higher in cases with moderate trauma (14%) than in those with severe trauma (5.3%).

In more than 98 per cent of the material the case history permitted an evaluation of health prior to fracture. Thus, 42 per cent of these cases were found to suffer from pertinent disease; disease was most common among men with moderate trauma (57%) and least common among men with severe trauma (21%). The validity of these findings is emphasized by the fact that in the entire material no group had a higher mortality rate than men with moderate trauma and previous disease (25%), and none had a lower mortality rate than previously healthy men with severe trauma (2.4%). Therefore, following moderate trauma, mortality was somewhat higher among men (17%) than among women (13%). Following severe trauma the reverse was found (men, 3%; women, 8%).

Throughout life the incidence of fracture of the neck of the femur in women doubled

TABLE 3. FEMALE FRACTURE RATE IN MALMÖ AROUND 1955

Age Group	Annual Fracture Rate per 10,000: (a) Neck of Femur	(b) Distal End of Forearm	Ratio (b/a)
50–54	4	28	6 8
55–59	7	56	7.8
60–64	12	59	4.8
65–69	22	69	3.5
70–74	39	50	1.3
75–79	52	58	1.0
> 80	117	59	0.5

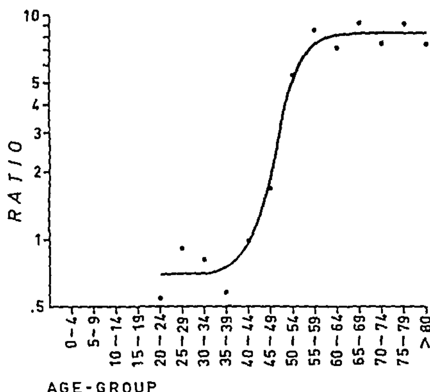


FIG. 1. Ratio women/men of age-and-sex specific rates of incidence of fractures of the lower end of the forearm.

approximately with each 5-year increment in age (Table 3 & Fig. 2).

FRACTURE OF LOWER END OF FOREARM

Fracture of the lower end of the forearm is defined here as comprising all fractures of the distal end of the radius and/or ulna up to 3 cm. above the wrist joint.

Fracture of the lower end of the forearm constituted 83.3 per cent of all fractures of the forearm in adults. In the lower age groups (Table 4) a slight preponderance of

TABLE 4. FRACTURE OF DISTAL END OF FOREARM IN MALMÖ 1953-1957

	Men				Women			
	Degree of Trauma			Total	Degree of Trauma			Total
Age Group	Slight	Severe	Unknown		Slight	Severe	Unknown	
20–24	8	9	3	20	2	8	2	12
25–29	2	9	2	14	10	3	2	15
30–34	5	14	4	23	12	5	3	20
35–39	5	9	14	28	11	5	1	17
40–44	13	16	5	34	24	10	1	35
45–49	12	17	1	30	37	15	4	56
50–54	7	8	2	17	77	24	1	102
55–59	7	7	4	18	138	25	8	171
60–64	7	9	2	18	126	19	10	155
65–69	8	6	—	14	133	16	9	158
70–74	7	2	1	10	76	16	1	91
75–79	5	1	—	6	57	11	4	72
80–84	4	1	—	5	28	2	3	33
85–89	—	—	—	—	13	4	1	18

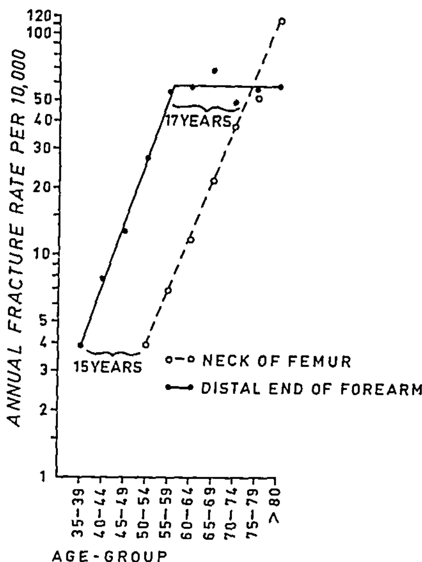


FIG. 2. Semilogarithmic plot of age-specific rates of incidence in women of fractures of lower end of forearm and of upper end of femur.

males was found. At about the age of 45, the ratio of females to males rose dramatically to between 7 and 8 to 1 (Fig. 1).

In 93 per cent of the cases an evaluation of degree of trauma could be made. In women, moderate trauma predominated over severe trauma by 2.1 before, and 5.5 after, the age of 50. In males, the corresponding figures were 0.6 and 1.3.

The age-specific incidence of fracture of the lower end of the forearm in women did not continue to rise in the older age groups (Table 3). The rate at which the incidence of fracture of the forearm increased in age groups below 60 seemed to be similar to that of fracture of the neck of the femur in age groups above 50 (Fig. 2). The lag between the two curves was 15 to 17 years.

DISCUSSION

In 1824 the cause of fracture in the aged was discussed by Sir Astley Cooper in his *Treatise on Dislocations and on Fractures of the Joints.*[6] He suggested that the skeleton became brittle because of changes with age in skeletal metabolism (Fig. 3). Although it lacks the term *osteoporosis*, Cooper's presentation sounds familiar to the reader of today[5,7,10] His ideas were amply confirmed by many studies of fracture epidemiology published during the 19th century. Thus, in 1882, the general pattern of the age and the sex specific incidence of various types of fractures was formulated clearly by Bruns[2] in an impressive review of fracture epidemiology. Important contributions to this literature were published recently by

Old age, however, is a very indefinite term; for in some it is as strongly marked at sixty, as in others at eighty years. That regular decay of nature which is called old age, is attended with changes which are easily detected in the dead body; and one of the principal of these is found in the bones, for they become thin in their shell, and spongy in their texture. The process of absorption and deposition varies at different periods of life; in youth the arteries, which are the builders of the body, deposit more than the absorbents remove, and hence is derived the great source of its growth. In the middle period of life the arteries and absorbents preserve an equilibrium of action, so that, with a due portion of exercise, the body remains stationary; whilst in old age the balance is destroyed, because the arteries act less than the absorbents, and hence the person becomes diminished in weight; but more from a diminution of the arterial than from an increase of the absorbent action. This is well seen in the natural changes of the bones, their increase in youth, their bulk, weight, and little comparative change during the adult period, and the lightness and softness they acquire in the more advanced stages of life; hence the bones of old persons may be cut with a pen-knife, which is incapable of making any impression on those of adults. Even the neck of the thigh-bone in old persons is sometimes undergoing an interstitial absorption, by which it becomes shortened, altered in its angle with the shaft of the bone, and so changed in its form as to give an idea, upon a superficial view, that it has been the subject of fracture, thus leading persons into the erroneous supposition, that the bone has been partially broken and re-united; but it requires very little knowledge of anatomy to distinguish in the skeleton, the bone of advanced age from that of the middle period of life.

FIG. 3. Facsimile from Sir Astley Cooper: Treatise on Dislocations and on Fractures of the Joints, ed. 4, p. 109, London, 1824.

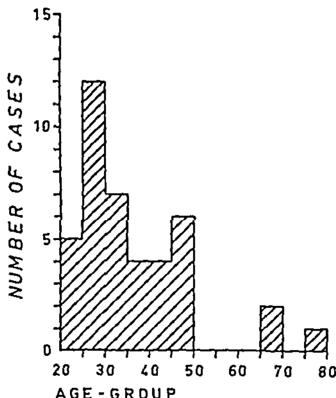


FIG. 4. Age distribution of adult cases of fractures of shaft of femur in men treated in the Malmö area from 1909 to 1917. (Based on data from Carlsson, P.: Om behandling av brott på lårbensskaftet, p. 17, Academic Thesis, Malmö, *1918*)

Stewart[8,9] and by Buhr and Cooke.[3] These studies show that the rate of incidence of fracture of the neck of the femur in the aged increases throughout life.

The reason for the increased risk of fracture in the aged may be attributed partly to factors outside the skeleton. Buhr and Cooke[3] and Boucher[1] have reviewed evidence of the greater tendency of aged people to fall because of a number of factors such as failing eyesight, impaired co-ordination, diminished muscular strength and other common defects in the aged.

For several reasons a change in the quality of the skeleton seems to be the dominating factor in fracture of the neck of the femur:

1. Fracture of the neck of the femur is hardly ever encountered in young healthy people, even though accidents severe enough to cause fracture of the shaft of the femur or the tibia are more common among young than old people. Figure 4 shows the age distribution of 43 cases of fracture of the femur in adult males published by Carlsson.[4] This unselected series was observed in the Malmö area during a 9-year period (1909-1917); i.e., at a time when automobiles were rare. It is presented for comparison with fracture of the neck of the femur, because even to-day in Malmö this latter fracture is rarely caused by automobile accidents. Similarly, fracture of the tibial condyles is common in the aged following even slight injury, while in young people it occurs almost exclusively following severe injury.

2. Our data on fracture of the neck of the femur show that complicating illness was less common among those who had had severe trauma than those who had had moderate trauma. The mean age of previously

healthy subjects with fracture of the neck of the femur was lower in those with severe trauma than in those with moderate trauma. Thus the degree of trauma necessary to fracture the neck of the femur would decrease with age, and illness seems to diminish the risk of meeting with severe trauma. Also, fracture of the lower end of the forearm was caused by diminishing degree of trauma in the higher age groups.

3. Our data showed that the rate of incidence of femoral neck fracture increased with age, relative to fracture of the distal end of the forearm (Table 3). This finding is supported by the data shown in Figures 4 and 15 in the article by Buhr and Cooke.[3] One may assume that the incidence of fracture of the distal end of the forearm should be roughly proportional to the product of the degrees of skeletal fragility and of trauma. The relative increase with age in incidence of fracture of the femoral neck could then be interpreted as evidence of progressive fragility of the femoral neck, other factors having been eliminated largely.

The dramatic rise at the age of the menopause in the female as compared with male incidence of fracture of the distal end of the forearm (Fig. 1) at the same age suggests strongly that endogenous[3,10] rather than dietary factors are the chief cause of the progressive skeletal fragility. Cooper[6] stated that during 30 years of active practice in London he had seen only two cases of fracture of the neck of the femur in subjects below the age of 50, and one of those, a female, had an aneurysm of the iliac artery. Considering that the food in London in the early 19th century most probably was more deficient—for example, in calcium—than that eaten in Oxford or Malmö more than 100 years later, it does not seem probable that calcium-deficient food is a major factor in fracture in the aged.

However, excepting evidence of loss of inorganic and organic constituents from the skeleton, objective symptoms of disturbed skeletal metabolism are rarely found in the individual case of fracture of the neck of the femur.[10] The lag period of 15 to 17 years between the rise in incidence of fracture of the lower end of the forearm and that of fracture of the neck of the femur (Fig. 2) might possibly indicate that symptoms of disturbed skeletal metabolism should be looked for one or two decades prior to fracture of the neck of the femur.

CONCLUSION

In this series of fractures of the neck of the femur, 41 per cent of the subjects were found to suffer from severe disease such as arteriosclerosis, hypertension with or without hemiplegia, and polyarthritis. In several case histories, disease was a certain (cancer metastasis), probable (polyarthritis) or possible (hemiplegia) major factor in the onset of fracture. A closer analysis of such cases may give a clue toward preventing fracture of the neck of femur. In the majority of the women, however, manifest disease or severe trauma did not seem to be important operating factors. This is an example of a group of patients in whom future research might reveal that fracture in the aged is a symptom of preventable metabolic disease.

To the present author it seems probable, as regards this clinical problem, that the contributions to bone physiology made by Franklin C. McLean will prove to be of immediate importance.

SUMMARY

In an urban population of more than 200,000 the age-and-sex specific rates of incidence in adults were determined for fracture of the neck of the femur and the distal end of the forearm The determinations were based on material over a 10-year period of 956 fractures of the femoral neck and material over a 5-year period of 1,192 fractures of the distal end of forearm. It was found that throughout life the incidence in women of fracture of the femoral neck doubled with each 5-year increment in age, while the incidence of fracture of the distal end of the

forearm did not change appreciably after the age of 60. Below the age of 40, fracture of the distal end of the forearm was somewhat more frequent in men than in women, while above the age of 60 women outnumbered men by a factor of more than 7.

The data are interpreted as showing that fracture in the aged primarily is a symptom of endogenous fragility of the skeleton.

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Epidemiologia de Fracturas in Subjectos de Etate Avantiate; Un Investigation Preliminari del Etiologia de Fracturas

Summario in Interlingua

In un population urban de plus que 200.000 subjectos, esseva determinate inter subjectos de plus que 20 annos de etate le distribution per etate e sexo de fracturas del cervice femoral e del termino distal del antebracio. Le casuistica consisteva de 956 fracturas del cervice femoral colligite in le curso de 10 annos e de 1.192 fracturas del termino distal del antebracio colligite in le curso de 5 annos. Esseva constatate que in feminas le incidentia de fracturas del cervice femoral es duplicate con omne quinquenne augmento del etate, durante que le incidentia de fracturas del termino distal del antebracio non se altera appreciabilemente post le etate de 60 annos. Infra le etate de 40 annos, fracturas del termino distal del antebracio esseva levemente plus frequente in homines que in feminas, durante que post le etate de 60 annos, feminas excedeva homines in isto per un factor de plus que 7.

Le datos es interpretate como prova que fracturas in subjectos de etate avantiate es primarimente un symptoma de fragilitate endogene del skeleto.

23

The Calcium Requirement of Man As Related to Diseases of the Skeleton

Ragnar Nicolaysen*

When asked to contribute to the volume in honor of our great colleague and friend Franklin McLean, it was felt that perhaps the views of an old worker in the field of calcium physiology might be of some interest. The title of the chapter was chosen to indicate that deficiency diseases rightly play an important role in the evaluation of nutritional requirements. The establishment of balance or of saturation with a given nutrient has little physiologic meaning in itself. Correlations with physiologic functions or frank disease are fundamental in the evaluation. In this contribution, systemic skeletal diseases obviously secondary to vitamin or protein deficiency, to acidosis, or to malabsorption as seen in fatty diarrheas need not be discussed. Osteoporosis, provided that it is not one of immobilization, is the only systemic disease that is relevant.

It is not seen in younger people in western Europe or in the United States. The disorder is very common in old people, and clinicians with experience of such cases seem to feel that inevitably it is one of old age and not related at all to low calcium intake in earlier years. The existence of osteoporosis or nonoptimal skeletal development in younger people has even been questioned and almost denied, even in geographic areas where the intake of calcium habitually is low. Thus a clear-cut calcium deficiency disease never has been established in man. However, it is necessary to examine the criteria used, as well as other and varying approaches to the problem, and a critical evaluation will be given in the appropriate sections below.

A few words on some of the essential aspects of calcium physiology may be of value. From birth to adult age, man accumulates from about 1,000 to 1,400 Gm. of calcium in the body,[21] implying an average daily accumulation of 170 to 200 mg. of calcium. Periods of rapid growth will be associated with higher retention and vice versa, and adaptation will compensate when for a time retention has not been proportionate to the growth of the skeleton. In adult age, balance is maintained when sufficient calcium is eaten. In the long-term study of adult males in this laboratory,[17] balance was observed on the daily intake of about 900 mg. daily. When in balance, the men excreted in the urine from about 100 to about 400 mg. daily, and the urinary calcium appeared to be a "personal" constant over the period of observation. In consequence, the net absorption of one man excreting 100 mg. and another excreting 400 mg. daily in the urine was 100 and 400 mg., respectively. The response to "halving" the daily intake is discussed later; however, adaptation of absorption was a prominent feature.

The absorption is subjected to a dual regulation.[21,9] Vitamin D is the primary regulating factor of importance throughout life. The other factor, "the endogenous factor," hy [illegible] y a humoral factor, which will stro [illegible] te absorption in the pres-

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ence of vitamin D in the body but not in a vitamin D free body, is also active (when brought into play) throughout life. This factor is responsible for adaptation of absorption, and the effect of it must be considered at any stage in the evaluation of calcium requirement.

The more precise evaluation of the calcium requirement of man can only follow actual observations of man. However, animal experiments generally give most useful information, and it seems better first to give an account of the animal experiments, which I believe are those best suited to supplement the necessarily more limited observations of man.

YOUNG ANIMALS

Gershoff, Legg and Hegsted[8] studied a litter of 6 mongrel dogs over a period of 34 months at intake levels of 0.1, 0.6 and 1.2 per cent calcium in the diet, resulting in daily intakes of 0.63, 1.62 and 2.67 Gm. calcium daily. (The reported growth rate for those animals given the low and the high levels was about the same, about 7.5 Kg. in the course of 7 months, indicating a puzzling difference between the figures for percentage and actual daily calcium intake.) The daily retention in the dogs on low calcium was 0.56 Gm. The ash content of the fat-free bones is stated to be the same, and no difference in the parathyroid glands could be detected between the animals on high calcium and those on low calcium. The dogs "adapted" to the low calcium intake did not go into negative balance on intakes of 50 mg. calcium daily, and they did *not* go into strong positive balance at high calcium intakes. The inference is that, to a considerable extent, predicted calcium requirement for balance is a reflection of the previous dietary intake.

Bessey[3] placed groups of rats on varying levels of intake throughout their lifetime:

> At the highest levels of intake the animals brought their body calcium to the maximum adult percentage of body weight. As the levels were decreased, they still reached this final percentage, but a little later in adulthood. Finally, a level was reached at which the first generation of rats were unable to reach a normal percentage of calcium in their skeleton. Thus after a certain stage of maturity they seemed to have lost the chance of bringing the calcium up to the adult level.
>
> If the second generation of this latter group of animals were kept on this low level of intake, they managed to reach a normal body percentage of calcium. But they did this by an adaptation in which the skeleton is smaller than normal. They looked like perfectly normal creatures, but their bodies were short, although the percentage of body calcium was normal.

H. C. Sherman[23] and Sherman and Sherman[24] summarize a rich experience in rat growth and development on various levels of calcium in the diet as follows:

> When the calcium content of a diet which originally contained an amount slightly above that of minimal adequacy [0.2% of air-dry food] was approximately doubled, there resulted better development, a higher level and longer period of adult capacity and vitality, and longer life without increased incidence of calcification of arteries but with a better store of calcium in the skeletal system. When the percentage of calcium in the food was increased only to 0.34 per cent of dry solids, the increased adult life-expectation was well marked for the males and only negligible for the corresponding females, which latter, however, had reared many more young at the higher level of calcium intake. When all females were kept unmated, those receiving the higher of these two levels of calcium intake lived longer than those on the level but slightly above minimal adequacy. *Moreover, at still higher levels of calcium intake females both reared more young and lived longer.*

OLD ANIMALS

A number of studies have been reported at intervals that partly uphold the opinion that more calcium is needed for equilibrium in old animals and also support the view that osteoporosis results from an increased catabolism of bones. Henry and Kon[11,12] reported two rat studies with quite conflicting conclusions. In the first, it was postulated that 2-year-old rats needed 0.5 per cent calcium in the diet to be in equilibrium; in the second, the results indicated that 2-year-old rats given a relatively low calcium diet

throughout life not only developed a well-calcified skeleton but also remained in balance on a daily intake of 25 to 30 mg. calcium (the actual daily intake on the 0.13% calcium diet). *Bharucha and McCay*[4] reported calcium losses in rats that in part were excessive beyond ages of 500 days, and Liu and McCay[13] found that old dogs had substantial difficulty in maintaining calcium equilibrium. In the latest article[14] this view is modified somewhat. When old dogs were compared with adult dogs, no difference in calcium utilization was observed, and it was suggested that the old dogs possibly were not old enough. Nicolaysen,[20] in extensive studies of old rats until death (at about 30-33 months of age) observed that they maintained calcium balance remarkably well on very low calcium diets (6-14 mg. daily calcium intake).

HUMAN STUDIES

Children

Balance experiments on well-fed children on a liberal calcium intake indicate that they will accumulate the optimal amount for normal skeletal development from about 1 Gm. calcium daily. Of greater importance in our discussion are reports of much lower requirements than those usually recommended, and we will examine these more critically below. Nicholls and Nimalasuriya[19] observed in Ceylonese children on habitually low calcium diets that the calcium excretion reached very low values. Bones from adult Ceylonese skeletons were also analyzed, and it was concluded that they did not differ from European bones. However, the indigenous population is small and the total skeletal weight also seems to be lower than that of Europeans.[21] In recent years Walker and Arvidsson,[25] in South Africa, have reported a number of studies of primary interest resulting in the conclusion that human calcium requirement has been overestimated. Ribs from children and adults were analyzed chemically following fat extraction and were found to be normal. However, it is known now that matrix and salts form and dissolve simultaneously, and the term *halisteresis* belongs to history. When bone tissue dissolves or is not formed optimally, the shape of the bone is normal, and the volume of the bone is normal (provided that growth is not retarded); however, the trabecular network will be less dense, and eventually the cortex also will be thinner. The bony tissue is replaced by marrow with a high fat content, and, when fat is removed by extraction, only the protein part of the marrow remains. This will tend to minimize the difference between optimally and quite substantially demineralized bones; for example, analysis in our laboratory of two corpora vertebrae gave the following results:

	Per Cent Ash of Wet Wt.	Per Cent Ash of Dry Fat-Free Wt.
1.	23.1	47.2
2.	13.2	45.1

The fat content was 23.7 and 49.6 per cent, respectively, of the dry weight.

The table on page 229 will be of interest in this connection. It seems to indicate that, in most cases, substantial differences in ash content of bones will be revealed by the aid of analyses of fat-free bones, whereas smaller differences may be obscured. There can be no doubt that the stepwise analyses give more complete information with regard to changes in bone composition and total mineral content. Since the analyses of fat-free bones have been used to indicate optimal mineral content, the above words of warning are in order.

In another article[26] serum calcium analyses from various groups of people are reported. In two groups of Bantu boys, values observed in other parts of the world in people on calcium-rich diets were found. However, in serum from adult South African Bantus, values roughly one tenth lower (range 7.9-10.9 mg.%) were observed. The authors contend that good health may be en-

ANALYSIS OF BONES FROM 12 OLD PEOPLE WITH AVERAGES FOR SIX IN EACH GROUP

	PER CENT ASH OF		
	WET WT.	DRY FAT-FREE WT.	PER CENT FAT
Rib			
Low group	24.8	53.7	33.0
High group	33.0	57.0	24.8
Difference %	27	6	32
Corp. vertebrae			
Low group	11.4	41.8	32.9
High group	17.6	46.8	22.2
Difference %	35	11.7	35
Os ilium			
Low group	21.2	53.5	30.7
High group	28.4	55.0	18.8
Difference %	25	2.7	38

joyed in spite of low serum calcium levels. This is also based on observations of fracture incidence and healing said to be the same as in people living on calcium-rich diets. Hirsch observed recently that the breaking strength of the collum femoris depended chiefly on the cortical part; the removal of the trabecular part had little influence. According to work by Ellinger *et al.*[6] on rats, calcium losses from the bones start in the trabecular part, but, when most of this has disappeared, substantial parts of the cortical bone also have been dissolved. Consequently, I would interpret the South African work as indicating that relatively clear evidence has been produced to support the view that the diet in the said area contains too little calcium to allow of optimal skeletal development. Rickets is said to be rare, due to abundance of sunlight. Therefore, the results indicate that calcium deficiency is responsible for the somewhat lowered serum calcium In any case, I consider that any decrease from normal in serum calcium is a strong indication of a deviation from normality that one should do everything possible to correct.

Attention should also be focused on the much-quoted study of Aykroyd[2] in India Indian schoolchildren (adequate control group established) benefited by additional calcium in the diet, as indicated by increased rate of growth in height as well as increased body weight; the increased food intake must be interpreted as consequent on stimulated growth.

ADULTS

Great numbers of balance studies have been conducted in the course of years in adults, and I will not review them. Only those that I feel to be highly relevant to the present short discussion will be referred to. It is quite obvious that the power of adaptation to various levels of intake will be our central problem, and only balance studies in which this specific factor has been taken into account and studied need more detailed reporting. Only three separate contributions will be used for the discussion.

McCance and his co-worker, Elsie Widdowson,[16] in the course of World War II studied the calcium balance in 8 persons with the specific purpose of elucidating calcium requirement relative to the phytic acid content of the diet. Their studies covered a period of 9 months, but no report of the details for the 9 months is given. I shall rely on the interpretation of the authors. Since then, McCance[15] has made very clear his view on adaptation by stating that no work has convinced him that adaptation to low calcium intakes takes place in animals or in man. He even says that he develops tetany when he goes on a brown-bread diet (regrettably no serum calcium analysis was

performed). In spite of the fact that adaptation as a factor of primary importance was established long before 1953, we cannot dismiss lightly the sizable Cambridge material. *It seems as if negative balances have been* quite common, and it is stated further that the balance in some persons deteriorated in the course of time. In one period, 2,000 I.U. vitamin D was given to each of the 8 persons, though with no measurable influence on calcium retention. The figures given for calcium requirement for balance on white and brown bread respectively were 734 and more than 1,140 mg. daily in 4 men, and 457 and more than 1,030 in 4 women. Obviously these figures are representative of careful observation over sufficiently long periods of time to justify their inclusion in any balanced consideration of our present problem.*

Hegsted *et al.*,[10] in their much-quoted and well-known study of the calcium balance in 10 long-term prisoners in the Central Penitentiary of Lima, concluded that the minimal calcium requirement of adult males was probably so low that deficiency was unlikely on most natural diets. Their contention is that figures of another type indicating much higher requirement reflect high calcium nutrition prior to the actual experiment. This is the reason for their discarding 1 of the 10 persons from their material. It is stated that he had received calcium injections at earlier dates. However, it is well known that the body excretes any such surplus in the urine; therefore, his urinary calcium might have been expected to be higher than in the others. This is not so; it is reported to be about 200 mg. daily. The mean estimated requirement (with the aid of linear regression calculations) was found to be 216 mg. daily with a range of 0 to 596 mg. (596 mg. in the said person who had been injected with calcium solutions). The equations derived were based on 7 to 10 periods of 6 to 10 days' duration in each individual at various levels of intake.

In the years 1949 to 1954 a long-term study of calcium balance in adult men was carried out in our laboratory, in order to investigate how adaptation could adjust men to various levels of calcium in the diet. A preliminary report was given in 1955,[18] and recently the full, very detailed report was published.[17] The program of the work was, *first,* to observe men over long periods of time (months to a year) on the present recommended daily allowance of about 800 mg. daily; second, to depress calcium intake as much as compatible with a normal Norwegian dietary; and, *third,* to observe adaptation and balance in the months to follow. Men at various ages were followed. Five men aged from 64 to 76 years retained from 5.5 to 43 Gm. calcium in 140 to 182 days, and, with a few exceptions of small negative balances, 39 men aged between 20 and 76 years were in balance or in positive balance on a mean intake of about 900 mg. daily. Successful observations at greatly reduced intakes (average 459 mg. daily) of 84 days and up to 532 days were made of 26 of the experimental persons. The time needed for adaptation varied considerably in those who again reached balance at reduced calcium intake. Some adapted instantaneously, others needed 9 months for adaptation, whereas a few did not adapt properly.

Following such observations, the calcium requirement for balance could again be calculated. Obviously, when a man adapts in the course of a few months to, say, 400 mg daily, following the loss of only a few grams of calcium, the implication is not that the minimum level has been reached. Anyhow, the result was that the majority of the men could adapt readily to the new, and in most cases halved, calcium intake. Thus the provisional figures *of calcium requirement per* Kg. (corrected) body weight of around 5

* A recent calcium-balance study for 95 consecutive days in 7 young men in the United States with a daily calcium intake of approximately 230 mg. indicated very little adaptation. The balance was negative, figures ranging from 28 to 120 mg for the last 16 days of observation (Thorangkul, D., *et al.*. J. Am Dietet A 35:23, 1959)

mg. were reached in a number of persons. Some caution was needed when such adaptation followed considerable positive balances at the high level of intake. The subjects were not in an optimal condition at the start of the experiment, and there is no method that can indicate how far from optimal such persons are, provided that x-ray routine examination does not reveal osteoporosis, because then it follows that the subject is already seriously demineralized. The reader is referred to the detailed report of Malm for a further scrutiny of this part of the material. To my mind much more attention should be paid to the few who gave indication of a high requirement following a real test of adaptation. Three cases are especially worthy of notice. One man (No. 551), 48 years of age at the beginning of the experiment, did not adapt at all. He was in balance in the course of 300 days on an intake of 890 mg. daily, but in the following 390 days of observation he was in a continuous negative balance. He absorbed very well, but his urinary calcium was about 350 mg. daily, and it was not reduced at all following the reduction of calcium intake. Consequently, his calculated requirement was 12.8 mg./Kg. body weight. Another man, 56 years of age, who retained about 25 Gm. calcium in the course of 210 days on 900 mg. calcium daily, needed 224 days to adapt to an intake of about 600 mg. daily; and, finally, one 31-year-old man, who retained 18 Gm. calcium in the course of 196 days (calcium intake 955 mg. daily), showed only slight indication of adaptation in the course of 224 days on 435 mg. daily. In fact, he lost nearly 100 mg. calcium daily on this diet.

Some additional observations may serve as a warning against any suggestion that No. 551 represents an abnormality that should not be regarded as part of a normal population. Statistically speaking, he was 1 in 1,000, and the 1 in 1,000 in a society should also be given attention. The calcium metabolism in these three persons did not differ significantly from that observed in others. The urinary level in all three cases, however, was horizontal and not influenced by halving the intake. This should be viewed bearing in mind that, on an average, only 30 mg. reduction of urinary calcium followed the halving of intake in the whole material. The urinary level was 230 mg. at 900 mg. calcium intake versus 200 at 450 intake level. By far the best-substantiated observation is that of No. 551, but the results in the other two men support the contention that, statistically speaking, he belongs to the same population.

Not a single person in this material was what others have seen fit to describe as poor absorbers. When two persons are in balance, say on 900 mg. calcium daily, and one excretes 100 mg. in the urine and 800 in the feces, and the other 400 mg. in the urine and 500 mg. in the feces, is, then, the low urinary calcium person a poor absorber? It is inherent in humans that some excrete much more calcium in the urine than others, and in balance they absorb much more. The argument would run equally well in the following way: (1) A person excretes high amounts of calcium in the urine; therefore, he absorbs high amounts of calcium. (2) A person absorbs very little calcium; therefore, he excretes very little in the urine.

The fact is that urinary calcium and net absorption are completely correlated in men in balance on liberal calcium intakes, but what correlates these two variables is not known. Perhaps the best way to express it is that they are subjected to an endogenous regulation of unknown nature or origin.

Let us next view the Hegsted, the Mc-Cance and the Oslo material together. As stated above, it seems unwarranted to exclude one important observation out of 10 based on successive balance experiments on the not very well-founded supposition that previous calcium injections were responsible. Anyhow, the Cambridge material had no calcium injections, and, according to the authors, considerable negative balances oc-

curred without any signs of adaptation. The Oslo material indicates that many persons need many months to adapt, whereas in the Cambridge material it seems that time was not allowed for adaptation to unfold its activity.

My temporary conclusion is somewhat as follows: South African observations indicate that human dietaries can be too low in calcium; the lowered serum calcium in adult Bantus is used as the main basis for such conclusion. Most adult persons can adapt to about as low a calcium intake as is commonly in use in the United States and most countries of Europe. Some persons seem to be definitely less adaptable than others and may, for optimal health and performance, need the present recommended daily allowance of 800 mg. or perhaps even slightly more. However, it remains to discuss frank calcium-deficiency disease; viz., old-age osteoporosis.

Balance experiments should also give indications in elderly people of increased catabolism of bone tissue. However, neither the Oslo material nor any other existing material is useful in this respect. Increased catabolism, in view of present-day knowledge of physiology of calcium metabolism, should appear in the form of increased urinary excretion. The problem was reviewed by myself and collaborators in 1953; Malm discussed it again in 1958.

When urinary calcium figures for men and women at various ages are compared, they seem to indicate no increased catabolism. And still very nearly all of us, myself included, strongly believe that old-age osteoporosis is a phenomenon of old age seated primarily in the skeleton and that it is not in the great majority of cases caused by lack of calcium in the daily food.

The question of malabsorption has not been discussed. In view of the fact that one group of authors—Ackerman and Toro—report excessive negative balances in some old persons due to high fecal calcium excretion, a few comments are needed. These individuals were all hospitalized, the periods of observations long enough to exclude sampling errors. However, fecal losses exceeding intake by 700 to 900 mg. daily are unique observations. In all instances these authors worked only with high calcium intakes, which must result necessarily in very low apparent absorption, and most probably the reported excessive loss reflects an unusual abnormality that does not throw any light on the development of osteoporosis. The simultaneous study of Bogdonoff, Shock and Nichols[5] in aged males concluded with the observation that the calcium balance at low levels of intake was identical with that in young adults.

In the course of the years I have considered repeatedly the paradoxic situation that no balance study or data for urinary calcium in such studies of elderly and old people indicate deviations from observations in younger age groups. The most reasonable conclusion seems to be that even long-term studies at various levels of intake do not constitute a method suited to reveal metabolic changes probably leading to osteoporosis.

Another method—the calcium load by slow intravenous infusion introduced by Schilling and Laszlo[22]—has been adopted and seems to have given results of considerable interest. J. M. Finlay *et al.*[7] used this technic in the study of various skeletal diseases. It seems as if the 4-hour skeletal retention calculation has given very useful information. In 15 patients with steatorrhea (with or without manifest osteomalacia), the retention was distinctly above that in normal controls, whereas the 4-hour retention was below normal in 7 out of 11 patients with osteoporosis and within the normal range in the others. Evidently the persons with secondary osteomalacia and those with osteoporosis represent two distinctly different groups. The first retain calcium avidly, indicating that excess matrix

ready to accept mineral salts is present. The second group can have no such excess matrix or no potentiality for forming such matrix. The situation, then, is distinctly different from the situation in a young body previously calcium starved, in which, when calcium (and phosphate) next is administered, the very high retention is characteristic. This situation also occurs in patients with hyperparathyroidism following removal of the adenoma; bone repair starts with great intensity. The conclusion seems clear: the results presented add weight to the view that old-age osteoporosis is not a calcium-deficiency disease. However, great care should be taken in accepting relatively few results as a proof of final and decisive value. The possibility still remains that the demineralization process in persons with osteoporotic tendencies can be slowed down with the aid of a diet rich in calcium and, what perhaps may be just as important, additional vitamin D. The remarkable retention tendency of the few old men observed in the Oslo material over a considerable period of time is the basis of the argument. Thus, there still is considerable need of further studies in this quite difficult field.

The sum of my views in the light of present-day knowledge may be given as follows:

A calcium-deficiency disease never has been clearly established. Most probably the calcium content in the diet of some geographic areas is too low in calcium to allow of optimal calcium nutrition. Considerable animal experimentation indicates that humans also should have a liberal calcium supply when it is at all possible, and it also supports the view that a daily recommended allowance of 800 mg. is not excessive for some adults. There is little evidence to indicate that osteoporosis as seen in old people is the result of malnutrition (calcium and vitamin D deficiency). A doctor encountering such cases can feel assured that he is doing neither better nor worse than usual in his evaluation of the possible origin when he concludes that calcium deficiency is not responsible. The possible role of additional calcium and vitamin D in the diet as prophylactic measures in the prevention of osteoporosis might be worth additional exploration.

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Le Requirimentos de Calcium del Homine in Relation al Morbos Afficiente le Skeleto

Summario in Interlingua

Il es solmente juste que le morbos de carentia ha un rolo importante in omne evalutation de requirimentos nutritional. Le establimento de un stato de balancia o de saturation pro le un o le altere nutriente particular es per se de pauc signification physiologic. Es postulate que correlationes con functiones physiologic o le presentia *manifeste de un morbo es essential in le* evalutation. Per consequente, le studio hic presentate include non solmente un evalutation de experimentos de balancia sed etiam de un varietate de altere datos, per exemplo investigationes in animales, analyses de factores geographic, e altere parametros que ha *a facer con le problema sub consideration.*

Un numero de studios in subjectos in stato de crescentia indica que le libere provision de circa 1 g de calcium in le dieta de juveniles es salubre sed que un adjustation a plus basse nivellos es prestemente effectuabile. Tamen, il remane certe dubitas con respecto al question de si o non il occurre un completemente normal o un optimal disveloppamento del skeleto in populationes que se mantene habitualmente a basse nivellos de calcium dietari. Quanto al adultos, le datos currentemente disponibile indica que le majoritate del subjectos pote adjustar se a un ingestion de calcium del basse valores que es usual in le Statos Unite e in grande partes de Europa. Il pare que certe *individuos non possede iste grado de* adaptabilitate e require, pro mantener un optimo de sanitate e de performance, le currentemente recommendate quota diurne de 800 mg. Tamen, le these es presentate que un morbo de carentia de calcium ha nunquam essite demonstrate e que il existe *nulle prova e pauc indicios que* le osteoporosis que es observabile in subjectos de etates avantiate es le resultato de malnutrition, i.e. de un carentia de calcium o de vitamina D in le dieta. Es notate que le rolo possibile de supplementos dietari de calcium e vitamina D como mesura prophylactic contra le disveloppamento de osteoporosis merita un re-exploration critic.

24

Osteomalacia, Osteoporosis and Calcium Deficiency*

B. E. C. Nordin, M.D., M.R.C.P., Ph.D.†

INTRODUCTION

Definition of Osteomalacia and Osteoporosis

Albright and his associates have made a great contribution to the understanding of metabolic bone disease in a series of articles, culminating in 1948 in the publication of a monograph[5] in which they re-emphasized the distinction originally laid down by Pommer in 1885[130] between osteomalacia and rickets on the one hand and osteoporosis on the other. Osteomalacia is a condition in which the ash content of the bone is reduced, and excessive amounts of uncalcified osteoid can be seen on histologic examination; rickets is the same condition in childhood. Osteoporosis is a disorder, or a group of disorders, in which there is a reduced amount of bone present in the skeleton; the ash content of such bone is normal, and osteoid borders are small or absent (Figs. 1 & 2). It is perfectly possible for the two conditions to be present at the same time.

Current Concepts of the Pathogenesis of Osteoporosis

The reduced bone mass seen in osteoporosis might be due either to increased breakdown of existing bone or to reduced formation of new tissue. Pommer suggested originally in 1885 that osteoporosis was due to impaired formation of new bone, and this concept was revived by Albright, Smith and Richardson in 1941,[6] when they declared that osteoporosis was a condition in which the osteoblasts did not lay down sufficient osseous matrix. This definition has since been repeated so frequently by its originators and others that it is commonly accepted as a statement of fact despite the paucity of evidence to sustain it. It is quoted as if beyond dispute by Follis,[54] Snapper,[151] Moldawer[107] and Bartter,[17] who invoke it to explain all types of osteoporosis, including that seen in Cushing's syndrome, in hyperthyroidism and in immobilization, as well as in the so-called postmenopausal and senile varieties. The negative nitrogen balance that may accompany the negative calcium balance in many of these conditions is held generally to support the Albright hypothesis, as also are the beneficial effects of estrogenic and androgenic hormones, which are thought to exert their action on the skeletal matrix.

It is not appreciated widely enough that this hypothesis rests on very slender foundations. The histologic assessment of osteoblastic activity is very difficult, but there is no convincing evidence that it is reduced in osteoporosis. The serum alkaline phosphatase, which is believed by Albright and Reifenstein to reflect the activity of the osteoblasts, is not reduced in any form of osteo-

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* Part of this work was done during the tenure of a Smith, Kline and French Traveling Fellowship at the Department of Medicine, Presbyterian Hospital, New York.

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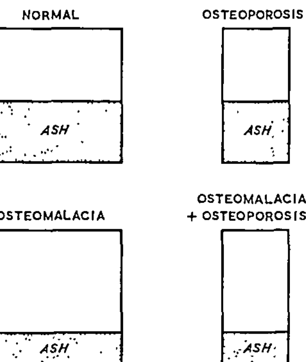


FIG. 1. Diagrammatic representation of the state of the skeleton in normal individuals, patients with osteoporosis, with osteomalacia, and with both together. (*Top, left*) The normal skeleton contains about 50 per cent ash. (*Top, right*) The osteoporotic skeleton (or bone) contains the same percentage of ash, but its mass is smaller In rickets and osteomalacia, theoretically it is possible for the bone mass to be normal, but its ash content is reduced (*bottom, left*). More commonly, however, there is a reduction in bone mass and in percentage ash (*bottom, right*). (Nordin, B. E. C : *In* Rodahl, K , *et al.*: Bone As A Tissue, New York, Blakiston Div., McGraw-Hill)

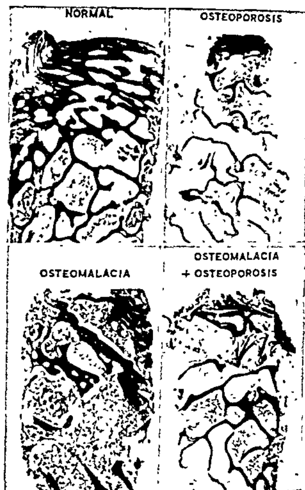


FIG. 2. Bone biopsies corresponding to the diagrams in Figure 1, all taken from the iliac crest 1 inch behind the anterosuperior spine (×15) (Nordin, B. E. C.: *In* Rodahl, K., *et al.:* Bone As A Tissue, New York, Blakiston Div., McGraw-Hill)

orosis except scurvy.[127] The causal relaonship between artificial menopause and steoporosis postulated by Albright has not een substantiated by Donaldson and Nasim;[45] nor is it likely that patients who evelop severe osteoporosis within a few ears of the menopause could have lost so nuch bone in so short a time—the process nust have started earlier.

Perhaps a more serious objection to the Albright hypothesis is that low protein diets o not appear to cause osteoporosis in aninals. Armstrong and Estremera[10] and Gardner[58] studied the effects of low protein diets on the bones of rats and mice, respectively. In both these experiments, the animals lost a considerable amount of weight, but the loss of bone weight was very small. The result was a rise in the bone weight/body ratio, which cannot be called osteoporosis.

The poor long-term results of conventional hormone therapy also suggest that the problem of osteoporosis is far from solved. Pain may be relieved by the administration of testosterone and diethylstilbestrol, but definite radiologic improvement has yet to be reported, even in patients observed for many years.

Osteoporosis may not be the "commonest of all diseases,"[78] but it is sufficiently com-

mon to be important; therefore, a reconsideration of current concepts appears to be justified. The present chapter is an attempt to review critically the data available on the pathogenesis of osteomalacia and osteoporosis and to suggest that some forms at least of osteoporosis are as likely to be due to negative calcium balance as to a primary disorder of the protein matrix of the skeleton. It will show that the rickets of vitamin D deficiency is probably not due simply to malabsorption of calcium and that deficiency of calcium does not produce rickets but osteoporosis in animals. The relevance of this to the etiology of osteoporosis in man will be discussed in relation to the current view that osteoporosis is a disorder of the bone matrix.

THE PATHOGENESIS OF RICKETS AND OSTEOMALACIA*

The Serum Calcium × Phosphate Product

It is agreed that rickets and osteomalacia are caused by a deficiency of vitamin D, although the precise sequence of events that leads to the development of the biochemical and clinical picture is not completely understood. In 1922, Howland and Kramer[75] showed that when the product of the serum calcium and phosphate concentrations in mg. per 100 ml. was less than 30, active rickets was present; when it was more than 40, rickets was either absent or healing. The relationship of this product to the solubility product of calcium phosphate is not clear, but this empiric rule has, as Hodge[71] observes, stood the test of time, although it would probably be more correct to consider the product $Ca^3 \times P^2$.[116] It has since been shown that rachitic rat cartilage will calcify in normal but not in rachitic serum,[34,134,145,168] and from this and other evidence it is inferred that it is the low calcium and phosphate levels in the serum that are responsible for the inadequate calcification of osteoid in rickets. This is the opinion of Park[129] and, in fact, was predicted by Pommer in 1885,[130] when he suggested that something "outside the bone" was at fault.

The only consistent exceptions to the rule of the Howland and Kramer product are provided by the renal rickets of glomerular insufficiency, in which osteoid borders and rachitic epiphyses develop in the face of a high product of circulating calcium and phosphate,[52,133] and by the rickets of hypervitaminosis D,[51,63] in which renal failure also appears invariably to be present. Yendt *et al.*[165] have shown that there is some factor present in the ultrafiltrate of nephritic serum that prevents calcium of rachitic cartilage in vitro, even at calcium and phosphate levels that normally are more than sufficient for calcification. It is conceivable that this factor might be citric acid, the serum level of which rises in uremia.[129]

The Role of the Parathyroids

The pathogenesis of rickets, therefore, is to be sought in the reduced product of serum calcium and phosphate that may accompany a deficiency of vitamin D. This reduced product is due most commonly to a fall in the serum phosphate concentration,[74] which might be attributable to impaired absorption of phosphate from the gut or increased excretion in the urine. Vitamin D, however, does not have any direct effect upon phosphate absorption,[34,60,111] and the fecal phosphate in osteomalacia may be relatively normal, whereas the urine phosphate usually is high.[5,89] This combination of a low serum phosphate concentration with a high rate of urinary phosphate excretion yields a raised phosphate clearance[104,115,120] and suggests that presence of parathyroid overactivity in osteomalacia. The raised phosphate clearance can be reduced by the infusion of calcium,[119] a procedure that is believed by many workers to suppress parathyroid gland activity.[73,86]

* References to rickets and osteomalacia in this chapter do not necessarily apply to so-called vitamin-D-resistant rickets



FIG. 3. Osteitis fibrosa with osteomalacia. Note the wide osteoid borders. (Lang, F. J.: Beitr. path. Anat. 87:143)

This indirect evidence of parathyroid overactivity in rickets is supported by the hypertrophy of the parathyroid glands that has been reported in vitamin-D-deficient animals[49,63,70,113] and humans.[18,48,69,88,125,135,150] Davies *et al.*[41] have reported radiologic evidence of osteitis fibrosa in osteomalacia and Lang[87] histologic evidence (Fig. 3).

Rickets may also be the end-result of any other process that lowers the serum phosphate. Dent[42] has postulated a "renal leak" of phosphate to explain some types of vitamin-D-resistant rickets, and in this he agrees with Fanconi.[50] Moreover, experimental rickets is produced most easily in rats by a low phosphorus diet that does not induce parathyroid hyperplasia[63] and is effective even after parathyroidectomy.[69] However, the available evidence suggests that in spontaneous rickets due to deficiency of vitamin D, the reduction in serum phosphate by parathyroid overactivity is an important, if not an essential, factor.[138]

Mellanby[97] showed that rickets was due to deficiency of an accessory food factor, which later came to be known as vitamin D. Subsequently it was established that the vitamin promoted calcium absorption,[153] and this has since been amply confirmed.[21,88,102,111] The idea that malabsorption of calcium is the cause of rickets has been for many years a "constantly recurring theme,"[27] and Albright and Reifenstein[5] believe that this malabsorption lowers the serum calcium and

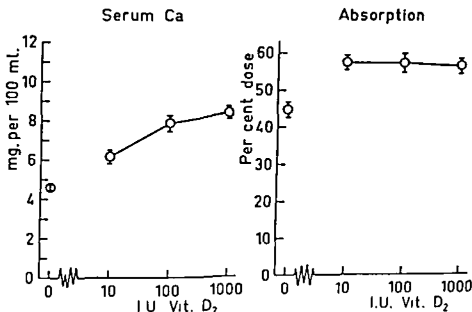


FIG. 4. The serum calcium and absorption of Ca^{45} after varying doses of vitamin D_2 to rats on low calcium diet.

Symbols indicate mean ± standard error of the mean.

The number of rats in the control, the 10 I.U., the 100 I U. and the 1,000 I U. groups was 10, 9, 4 and 6, respectively.

(Carlsson & Lindquist: Acta physiol. scandinav. 35:55)

so stimulates the parathyroid glands. A natural corollary would be that dietary calcium deficiency similarly lowered the serum calcium and produced rickets, and it is clear that many workers besides Albright believe this to be the case.[82,170] In fact, it is widely stated that rickets and osteomalacia may be due to deficiency of vitamin D or of calcium, or of both.[43,163,166] However, experimental work to be reviewed on pages 242 to 244 shows that calcium deficiency in fact causes osteoporosis in animals and not osteomalacia and, therefore, suggests that vitamin D must exert some other essential action besides the promotion of calcium absorption.

THE ACTIONS OF VITAMIN D AND OF THE PARATHYROID HORMONE

The Effect on the Skeleton

McLean pointed out in 1941[93] that the effect of calciferol on the serum calcium and phosphate in rickets could not be adequately explained by improvement in absorption alone. He attributed the "calcemic" action of vitamin D to the mobilization of bone salt. This concept is supported by the work of Carlsson and Lindquist,[35] who showed that 10 units of vitamin D given to rachitic rats on a low calcium diet produced maximal absorption of Ca^{45} with a significant rise in serum calcium; doses of 100 and 1,000 units caused further significant increases in the serum calcium without any further improvement in absorption (Fig. 4). They concluded that the effect of vitamin D on the serum calcium level in these animals could not be adequately explained by improved absorption of calcium. Carlsson[34] has also reported that the serum phosphate of rats on a phosphorus-free diet was significantly higher in those receiving vitamin D than in those that were vitamin D deficient, and that starvation caused hypocalcemia in vita-

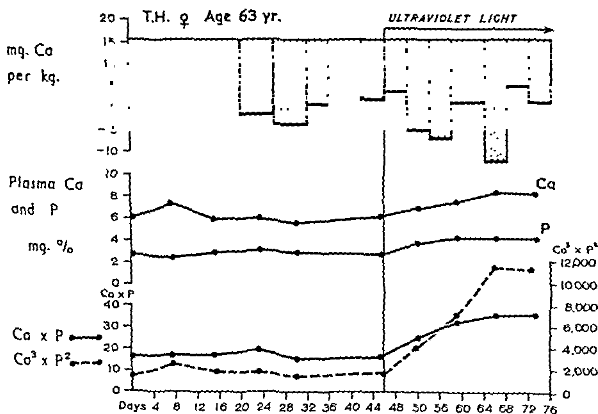


Fig. 5. Calcium balance, plasma calcium and phosphate and calcium phosphate product in a patient with steatorrhea and osteomalacia treated with ultraviolet light. Note that the plasma product rises although there is no change in calcium balance. (Nordin, B. E. C.: Clinical Endocrinology, New York, Grune)

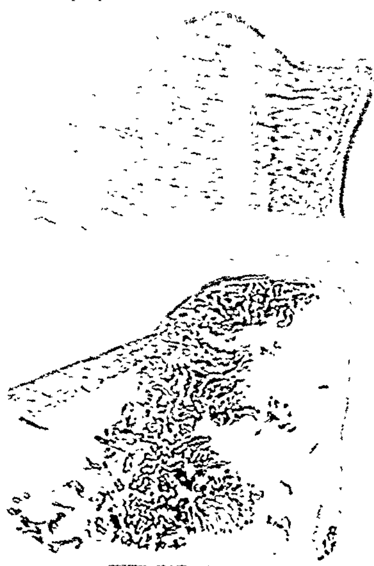


FIG. 6. Epiphysial cartilage o[f] a dog given a low calcium die[t] until its bones were fracturin[g] (*top*) compared with the same region in a dog with spontaneous rickets. (Miwa & Stoeltzner: Beitr. path. Anat. 24:578)

min-D-deficient rats but not in rats given vitamin D. In these two experiments, absorption effects were eliminated by the exclusion of phosphate and calcium, respectively, from the diet; therefore, the mineral in the serum must have come from bone. Mellanby[8] has stated that when vitamin D is given to rachitic rats with inadequate dietary calcium, the rickets may be cured, but at the expense of the mineral content of the shaft of the bone.

Figure 5 shows data on a patient with steatorrhea and osteomalacia that point the same lesson. The low Ca × P product was restored rapidly t[illegible] by ultraviolet light before there [illegible] ...ment in calcium balance.

These data indicate that vitamin D raises the serum calcium by a direct action on bone and are compatible with the known effect of vitamin D overdosage, in which hypercalcemia cannot be attributed to excessive absorption of dietary calcium, and the bone lesion may be an osteitis fibrosa similar to that seen in primary hyperparathyroidism.[8,11,29,...]

The [illegible] hormone also raises the serum [illegible] a direct act[illegible] bone.[16,...] [illegible] has [illegible] action [illegible] on the [illegible] tween [illegible] calcium [illegible] miner[illegible] pointed out presen[illegible] 'd horm[illegible] librium [illegible] at a s[illegible]

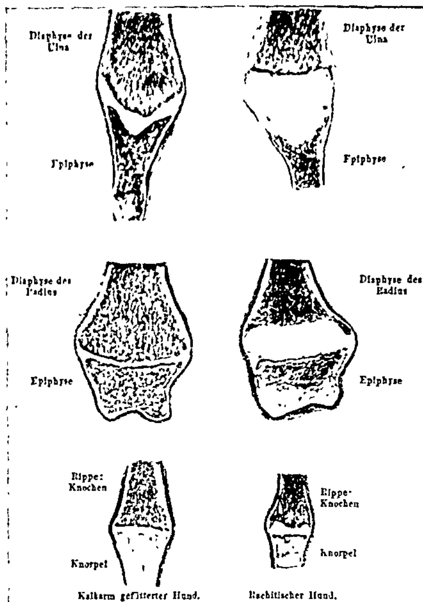


FIG. 7. Epiphysial regions in a dog fed a low calcium diet until its bones were fracturing (*left*) compared with the corresponding regions in a dog with spontaneous rickets (*right*). (Reimer & Boye: Zentralbl. inn. Med. 26:953)

about 10 mg. per 100 ml., whereas after parathyroidectomy it falls to about 6 mg. per 100 ml. It could now be added that vitamin D is probably also concerned in the maintenance of this equilibrium and that the effect of vitamin D deficiency on the serum calcium of hypoparathyroidism can be corrected by the administration of calciferol. The normal serum calcium appears to be the result of their synergistic action

Effect on Urinary Phosphate Excretion

The action of the parathyroid hormone in promoting the renal excretion of phosphate has already been mentioned. Vitamin D is also believed to possess this action, but Albright and Reifenstein[5] believe that it is less potent than the hormone in this respect. This is borne out by the relative inefficacy of calciferol in lowering the serum phosphate concentration in patients with hypoparathyroidism. It is this difference in their phosphaturic action that explains why parathyroid overactivity causes rickets whereas vitamin D cures it.

Effect on Calcium Absorption

The effect of vitamin D on calcium absorption is well recognized and has been discussed. The alleged effect of parathyroid hormone on calcium absorption is not well

documented and should be accepted with reserve.

EXPERIMENTAL OSTEOPOROSIS IN ANIMALS

Dietary Calcium Deficiency

The fact that vitamin D exerts a direct action on the skeleton explains why the effect of calcium deficiency differs from that of vitamin D deficiency, as will now be shown to be the case.

Attempts to produce rickets in animals by dietary deficiency of calcium date back to the middle of the last century. Skeletal lesions of a kind were produced by these diets, but, in 1874, Weiske[160] was able to show that the ash content of the bones was not reduced; consequently, the condition was not rickets.

Further progress had to await the publication, in 1885, of Pommer's monograph, in which he showed for the first time that the characteristic histologic feature of rickets was the wide uncalcified osteoid border. In the next three decades, many articles were published on the subject in Germany, most of them showing that low calcium diets produced osteoporosis. Miwa and Stoeltzner[106] examined the bones of a dog that had been fed a low calcium diet for 6 weeks. The bones were thin and fragile, but the epiphyses had not widened, and no wide osteoid borders were seen (Fig. 6). Similar results were reported by Reimer and Boye[134] (Fig. 7) and by Gotting.[59] Some workers considered that the bone lesions of calcium deficiency were due to impaired bone formation, but the majority favored an increased rate of removal,[44,59,122] and some favored both.[57] All this early work was excellently reviewed by Pommer in 1925,[131] when he concluded that low calcium diets caused osteoporosis, not rickets.

More recent work confirms these German observations Bauer *et al.*,[19] in 1929, showed that low calcium diets led to resorption of bone trabeculae in cats. They did not publish microphotographs, but they made no mention of rachitic lesions; nor was there any suggestion of rachitic changes in the gross anatomy. Jaffe *et al.*[81] found that low calcium diets produced osteoporosis in dogs, and Bell *et al.*[22] produced osteoporosis in rats by feeding them diets containing less than 0.36 per cent calcium with adequate vitamin D (Fig. 8). Kintner and Holt[92] showed conclusively that equine osteoporosis in the Philippines was due to calcium deficiency. They called this disorder osteomalacia, but their data show that it was in fact osteoporosis. Carttar *et al.*[36] reported that rats kept on a limited calcium intake developed a skeleton that was "severely osteoporotic and slightly rachitic." Harrison and Fraser recently reported osteoporosis in rats on diets low in calcium but with adequate amounts of vitamin D (Fig. 9). Follis, reviewing the subject in 1948,[51] noted that low calcium rickets had been produced but that no descriptions were available in animals the diets of which contained optimal amounts of vitamin D. Schmidt[140] stated, "It is clear that rickets cannot be caused by calcium deficiency; this causes osteoporosis." This statement recently was confirmed once again in rats by Crawford *et al.*[39] It must be remembered that the low calcium type of rickets that can be produced in rats is obtained on diets lacking vitamin D.[63,146]

Malabsorption of Calcium

Osteoporosis has also been produced by artificially induced malabsorption of calcium. Bussaberger *et al.*[32] produced osteoporosis in puppies by gastrectomy, and their balance data showed that this procedure was associated with a great increase in the fecal calcium. Similary, Mellanby[94] stated that the administration to puppies of sodium phytate with adequate amounts of vitamin D did not cause rickets, but osteoporosis, and that this could be prevented by a high intake of calcium.

Relationship to Osteitis Fibrosa

Various forms of negative calcium balance appear to produce osteoporosis or

osteitis fibrosa, depending on the age of the animals and the rate of the bone destruction. Jaffe *et al.*[81] reported that, whereas simple low calcium diets produced osteoporosis in dogs, the addition of acid produced osteitis fibrosa, and they attributed this to a difference in the rate of bone destruction. Osteitis fibrosa could also be produced by dietary calcium deficiency in rapidly growing puppies. Large doses of vitamin D and para-

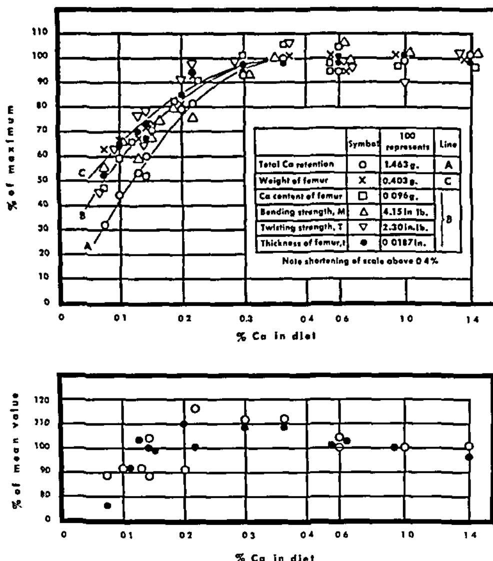


FIG. 8. The effect of varying the calcium content of the diet on the femora of rats.

(*Top*) Various findings, shown in the panel in the graph, are expressed as a percentage of their maximum value plotted against the percentage of Ca in the diet. (Note shortening of the scale above 0 4%.)

Shows that increasing the dietary calcium increased the weight and the strength of the femur until maximum values were obtained when the diet contained 0.36 per cent calcium.

(*Bottom*) Increase in weight of the whole animal and percentage of Ca in the whole femur, both expressed as a percentage of the mean value, are plotted against percentage of Ca in the diet. Increase in weight, O, 100 represents 159.2 Gm. Percentage Ca in whole femur, •; 100 represents 23 0 per cent.

Shows that the low calcium diets did not produce rickets, inasmuch as there was no significant change in the ash content of the bone on the different diets

(Bell, Cuthbertson & Orr: J. Physiol. 100:303)

thyroid hormone can both produce osteoporosis in animals,[14] but both can also produce osteitis fibrosa.[111] In both lesions it is the trabecular bone that is affected predominantly.[19,25,80]

DIETARY CALCIUM, SERUM CALCIUM AND THE SKELETON

It has been shown that there is a difference between the effect of calcium deficiency and that of severe vitamin D deficiency on the animal skeleton, the former causing osteoporosis and the latter rickets. The explanation is probably that the fall in serum calcium in vitamin D deficiency is due to lack of the calcemic action of the vitamin rather than to malabsorption of calcium. The serum calcium is not affected by simple dietary deficiency of calcium. It has already been pointed out that the tissue fluid minerals are presumed to be in a state of equilibrium with the crystals of a calcium phosphate compound in the bones. The surface area of these crystals is estimated at about 130 m² per Gm.[46] Therefore, the area exposed to the tissue fluid is very great, even if it represents only 5 per cent of the total bone mineral.[110] This must facilitate the rapid migration of ions into and out of the skeleton.

Hastings and Huggins[64] demonstrated the importance of the skeleton in sustaining the serum calcium when they showed that the rapid removal of calcium from the blood of anesthetized dogs had virtually no effect on the serum calcium concentration. This work has since been confirmed by Copp[38] and by Talmage and Elliott,[154] who have shown that ionized calcium removed from the circulation by other methods is replaced rapidly by calcium from the skeleton, even in parathyroidectomized animals. Therefore, it is probable that the small quantity of calcium absorbed from the diet plays a relatively minor part in determining the concentration of calcium in serum and that in dietary calcium deficiency the inevitable loss of calcium in the urine would be replaced by migration of calcium out of the skeleton and subsequent or simultaneous destruction of bone.

There is no reason to assume that the maintenance of the serum calcium in this way involves stimulation of the parathyroid glands, but there is very little direct information on the effect of simple dietary deficiency of calcium on the serum calcium and parathyroids. Low calcium diets are said frequently to cause parathyroid hyperplasia in rats, but all the experiments except one[91] were performed with diets deficient in vitamin D, and Crawford *et al.*[39] have shown that, if vitamin D is supplied, low calcium diets do not cause parathyroid hyperplasia. On the other hand, Benzie *et al.*[23] have reported a fall in serum calcium in sheep on low calcium diets; but, since there was a simultaneous rise in the serum phosphate, it appears that there was no compensatory parathyroid overactivity. Kintner and Holt[92] described a calcium deficiency state in horses in which the serum calcium was not reduced significantly.

It is known that short-term balances on a low calcium diet in man do not affect the serum calcium level,[3] and Malm[95] now has shown that the same is true of prolonged calcium deficiency. Malm, Nicolaysen and Skjelkvale[96] have shown that prolonged low calcium diets may have little or no effect on the excretion of calcium in the urine, and this suggests that there is no significant fall in the serum calcium. Three cases of osteoporosis following prolonged calcium deficiency have been reported,[2,13,20] and the serum calcium and phosphate levels were normal in each case.

Further studies are required of the effect of prolonged low calcium intakes on the serum levels of calcium and phosphate and on the phosphate clearance in man, but there is at present no evidence that calcium deficiency per se stimulates the parathyroids or leads to osteomalacia.

CLINICAL OSTEOPOROSIS

General Considerations

The osteoporosis produced in animals by dietary calcium deficiency must be regarded

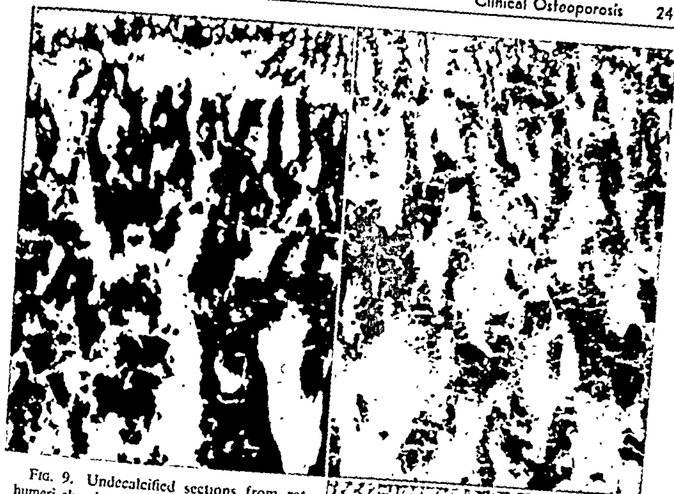


FIG. 9. Undecalcified sections from rat humeri showing normal control (*top, left*), effect of low calcium diet (*top, right*) and effect of vitamin-D-deficient diet (*bottom, right*). Note loss of bone on low calcium diet but absence of osteoid borders. (Von Kossa, ×90) (M. Harrison & R. Fraser)

as the result of a prolonged negative calcium balance in which mineral is mobilized from the skeleton to sustain the serum calcium. Because the serum calcium is sustained, the loss of calcium into the urine continues, and bone is destroyed progressively. This is in accordance with the current view that demineralized matrix always is removed.[36] There are two other possible causes of negative calcium balance besides dietary deficiency: malabsorption of calcium and hypercalciuria. Studies suggesting that malabsorption of calcium also leads to osteoporosis in animals have been quoted, and, in the final analysis, hypercalciuria is the cause of osteoporosis produced by administration of acid, vitamin D or parathyroid hormone, which have already been mentioned, since it is the way in which the organism loses its calcium.

It remains to be considered whether or not there is any evidence that negative cal-

cium balance could be the cause of clinical osteoporosis in man. The three possible causes of negative calcium balance mentioned above will be considered separately.

Dietary Deficiency of Calcium

Mitchell and Curzon[105] analyzed 139 calcium balances in normal individuals and found that the average requirement was about 10 mg. per Kg. per day. The data of Malm *et al.*[106] suggest a similar figure, although they interpret them rather differently, and the same requirement is postulated by Irving.[77] Although this is an acceptable mean figure, there is probably a wide range with considerable individual variations. Thus, Figure 10 shows the calcium requirement in 32 normal individuals as calculated from balances found in the literature at more than one level of intake; it ranges from 2 to 19 mg. per Kg. per day.

The average intake of calcium in the Western World is about the same as the average requirement, but this range is also very wide, and many surveys have disclosed large numbers of people living on much lower intakes.[30,124,136,165] It is evident that unless the individuals with high requirements happen to be those on high intakes, and those on low intakes have low requirements, there is a very real possibility that many people may be in negative calcium balance. In fact, it could be avoided only if man possessed the power to adapt to low calcium intakes.

The evidence of adaptation is conflicting. Hegsted and Moscoso[67] have expressed the view that adult men need little or no calcium, but this view was based on studies in Peru, where the inhabitants may have adapted to low calcium intakes over many generations. It is well known that individuals go into negative calcium balance when placed on low calcium diets for a short time, but the evidence from long-term balances suggests that most individuals are able to adapt to low calcium intakes. Walker *et al.*,[159] for instance, found that adaptation to *a 500 mg. intake was possible. On the other* hand, some data[95,96,112,162] show that some subjects at least are incapable of adaptation to low calcium intakes continued over very long periods. It may be significant in the pathogenesis of senile osteoporosis that

CALCIUM REQUIREMENT

40 OBSERVATIONS OF 32 SUBJECTS

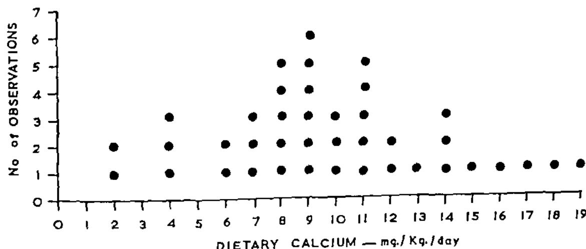


Fig 10 Calcium requirement of 32 normal subjects calculated from calcium balances at more than one level of intake. Note the wide range (Nordin, B. E. C.: *In* Rodahl, K, *et al.:* Bone As A Tissue, New York, Blakiston Div, McGraw-Hill)

elderly subjects seem to require more than the young to remain in balance.[1] It is probable, therefore, that some of the inhabitants of the Western Hemisphere are in negative calcium balance; in fact, it would be surprising if this were not the case. Clearly, the calcium that is lost must be taken from the skeleton, but neither osteitis fibrosa nor osteomalacia due to pure calcium deficiency has ever been reported. The few dietary surveys that have been carried out in osteoporosis have shown that many patients with this disorder have been on an inadequate calcium intake,[31,117,124,159] and at least three cases of severe osteoporosis have been reported in which the patients had been on particularly low calcium intakes for very many years.[2,13,20] Groen[91] has reported low calcium intakes in patients suffering from senile paradentosis. On the other hand, a dietary survey of 47 patients with fractures due to minor trauma proved to be inconclusive.[65]

Malabsorption of Calcium

It is difficult to assess calcium absorption in a given patient unless it is severely impaired, but in our experience it is sometimes reduced in osteoporosis; whether or not this is due to mild deficiency of vitamin D is not yet known. There are many studies that suggest that gastric acid may also be a factor in calcium absorption in so far as it influences the pH of the duodenum.[108] If this is correct, it may help to explain the osteoporosis that has been reported in association with achlorhydria[100] and after gastrectomy,[177] and the increasing incidence of achlorhydria with advancing years may have some bearing on the age incidence of osteoporosis. A series of balance studies in gastrectomized patients[142] suggested a mean calcium requirement as high as 26 mg. per Kg. per day. Probably many of these patients had steatorrhea. We have found steatorrhea in about 15 per cent of patients

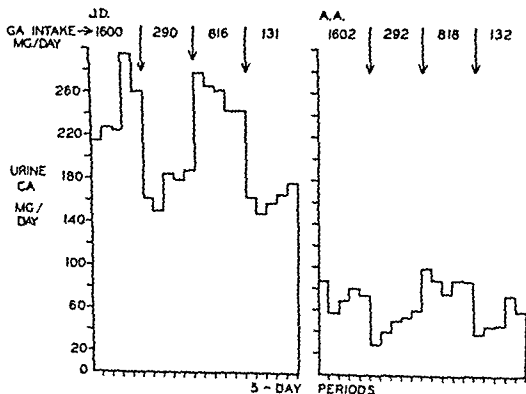


Fig. 11 The effect on urine calcium excretion of varying the calcium intake. The change from a high to a low intake was reflected in the urine calcium in each case, but the two subjects fluctuated around very different mean values. (Bogdonoff, Shock & Nichols. J. Gerontol. 8:284)

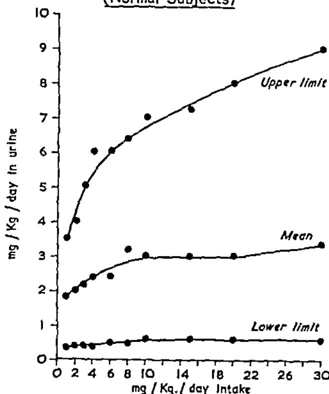


FIG. 12. Mean urinary calcium and lower and upper limits in normal subjects at different levels of intake. Note small change in mean excretion with changing intake. Calculated from Knapp.

with osteoporosis and regard them as examples of malabsorption of calcium.[117] This malabsorption is probably the result of vitamin D deficiency; therefore, it appears that in some circumstances steatorrhea causes osteoporosis and in others osteomalacia. The difference could be one of degree, osteomalacia being the result of a more severe deficiency of vitamin D and consequent fall in plasma calcium and phosphorus

Hypercalciuria

Very little is known of the factors that govern the urinary excretion of calcium, but it is clear from the data of Knapp[84] that very large changes in dietary intake are required to produce appreciable changes in urine calcium. The studies of Malm[95] suggest that the excretion of calcium is a characteristic of the individual rather than of the dietary intake of calcium. This is supported by the data of Nicolaysen *et al.*[112] and of Bogdonoff *et al.*[28] (Fig. 11). The data of Knapp (Fig. 12) also show how little relationship there is between dietary calcium and mean urinary calcium in normal people. In our experience there is no connection between dietary calcium and urine calcium in osteoporosis. Presumably individuals with a high urinary calcium are more liable to go into negative calcium balance than others; therefore, one might expect a certain proportion of patients with osteoporosis to have hypercalciuria if negative calcium balance were the cause of the condition. This is in fact the case.[79,117] Albright and Reifenstein[5] argue that hypercalciuria is a consequence of the inability of the skeleton to utilize available calcium, but it seems more likely that it is the cause of or a contributory factor to the disease, since it must predispose to a negative calcium balance.[114]

In elderly patients with osteoporosis the urine calcium tends to be normal or low. Some workers speak of the disease as being "inactive" in these cases. More probably the truth is that they have succeeded finally in adapting to their intake but too late to save their skeletons.

DISCUSSION

Primary Osteoporosis

The possibility that osteoporosis may be the result rather than the cause of negative calcium balance never has been considered seriously since Albright and his associates, in 1941, postulated that it was a disorder of the protein matrix of the bone; but it is probable that the Germans were aware of the relationship between osteoporosis and calcium deficiency before World War I. The discovery of vitamin D after the war and the subsequent demonstration of its action on the absorption of calcium inevitably suggested that rickets was caused by malabsorption of calcium and led to the assumption that calcium deficiency must cause the same lesion.[5] This review of the literature, how-

ever, has shown that calcium deficiency produces osteoporosis in animals and has revealed a number of reasons for believing that negative calcium balance due to dietary deficiency of calcium, malabsorption of calcium or hypercalciuria could explain clinical osteoporosis in man. Because of the popularity of the Albright doctrine at the present time, very few studies have been published that bear directly on this hypothesis, but, generally, our work so far appears to be compatible with it.

We have found[117] that the mean calcium intake of patients with osteoporosis is significantly lower than that of a comparable group of controls and that the mean calcium excretion is higher.

In the majority of the patients studied, calcium therapy without hormones suffices to achieve positive calcium balance[161] and relief of backache.[117] Furthermore, measurement of bone accretion rate with Ca^{47}(19) or Ca^{45}(66) has not shown any reduction in the rate of new bone formation in osteoporosis.

The sex incidence of osteoporosis requires consideration. Albright and Reifenstein[5] believe that estrogen deficiency leads to a high incidence of osteoporosis in postmenopausal women, but Donaldson and Nassim[45] were unable to confirm the suggestion that osteoporosis occurred earlier or more frequently in women after an artificial menopause. Pregnancy and lactation, on the other hand, might very well contribute to the development of osteoporosis if the condition were due to calcium deficiency, just as pregnancy is believed to be a contributory cause of iron-deficiency anemia in women.[164] Nordin and Roper[121] have reported four cases of osteoporosis in young women following pregnancy and have pointed out that virtually all the published cases of idiopathic osteoporosis in women have followed pregnancy. The present writer has since seen four more such cases; in his series of cases of osteoporosis, the average number of children per woman was three. It may be significant that no case of osteomalacia attributable to pregnancy has been reported in the Western Hemisphere, as might be expected if calcium deficiency was a cause of osteomalacia, and those cases reported by workers from China[101,132] could legitimately be regarded as suffering from acute osteoporosis (due to calcium deficiency) superimposed on chronic osteomalacia (vitamin D deficiency).

Dietary deficiency of calcium may not be common in the Western Hemisphere, but there is no reason to suppose that it does not occur. Sherman[143] claims that many American citizens are receiving an inadequate intake of calcium and shows that in 150 American dietaries the calcium intake ranged from 0.24 to 1.87 Gm. per day. It is scarcely conceivable that the subjects taking the lowest of these calcium diets should have been in calcium balance, and it is extremely probable that women, subject to the drain of pregnancy and lactation, would be more liable than men to show the effects of prolonged negative balance. However, we do not wish to rule out the possibility that there may be endocrine factors in the control of urine calcium and adaptation, but this is a very different matter from claiming a hormonal effect on the bony matrix.

The possible role of malabsorption of calcium in leading to negative calcium balance remains speculative in the absence of reliable studies on calcium absorption: at the present time vitamin D is the only factor that definitely is known to be important in this respect. In the osteomalacia of steatorrhea, however, which is believed to be a form of vitamin D deficiency, bone biopsy may reveal histologic evidence of osteoporosis (i.e., thin trabeculae), as well as of osteomalacia (i.e., wide osteoid seams). This has already been noted by Atkinson *et al.*[12] in the bone disease of chronic obstructive jaundice and has been seen by the present author in many other cases of osteomalacia due to steatorrhea (Fig. 2). These observations are compatible with the concept that osteoporo-

sis may be caused by negative calcium balance due to malabsorption, and, in fact, Hess[69] states that osteoporosis is seen in rickets only if the patient is in negative calcium balance. Thus, in these circumstances, vitamin D deficiency appears to contribute to the development of osteoporosis by interfering with calcium absorption. There is not much evidence to show whether minor degrees of vitamin D deficiency may or may not play a part in the development of pure osteoporosis, but certain observations point in this direction.[117] The role of gastric acid in calcium absorption remains obscure.

The animal work and the clinical observations that have been quoted suggest that hypercalciuria is a third factor that might contribute to negative calcium balance. It is well known that many patients with osteoporosis have a high urinary calcium, and this is compatible with the suggestion that hypercalciuria may predispose to osteoporosis. On the other hand, Albright and Reifenstein[5] believe that the raised urine calcium seen in osteoporosis is the consequence of the inability of the matrix of the bone to utilize available calcium, which is, therefore, shed in the urine. The observed facts are equally compatible with either explanation, but the former is inherently more probable.

Albright and Reifenstein consider that the hypercalciuria of renal tubular acidosis lowers the serum calcium, stimulates the parathyroids to lower the serum phosphate, and so leads to osteomalacia. However, in virtually all their published cases, as also in those of Pines and Mudge,[129] the serum calcium concentration was normal, and the low phosphate concentration should instead be attributed to the phosphaturia that may result from acidosis, as Milne *et al.*[104] suggested in a case of Fanconi's syndrome. However, this does not explain the parathyroid hyperplasia reported in one case of Pines and Mudge and one of Albright.

Albright and Reifenstein believe that "idiopathic hypercalciuria" also causes osteomalacia, but the only case that they describe was also suffering from steatorrhea, and no other work has ever shown that hypercalciuria per se can cause osteomalacia.

The histologic appearance of osteoporotic bone in most cases is equally compatible with impaired bone formation or a slightly increased rate of removal. Osteitis fibrosa, however, may be seen in hyperthyroidism (see below), suggesting that bone destruction is in progress, and conversely osteoporosis may be seen in primary hyperparathyroidism,[37,115] in which bone destruction is certainly the essential process. These considerations show that the same processes that cause osteitis fibrosa may also cause osteoporosis, and vice versa, and imply that the latter may be due to bone destruction.

If the hypothesis advanced in this chapter is correct, the therapeutic implication is obvious: patients should be treated with a high calcium diet. This should at least arrest the process. It is significant that a high calcium intake may alone suffice to produce a positive calcium balance in patients with osteoporosis.[7,117,161] Furthermore, good results were reported with calcium and vitamin D therapy before the present vogue for hormone treatment was established.[24,156] The subjective clinical improvement and the fall in urine calcium that can be produced with estrogenic and androgenic hormones need to be explained, but the evidence that these substances exert some direct effect on the matrix of the bone is unsatisfactory.

Secondary Osteoporosis

Hyperthyroidism

Negative nitrogen balance and consequent impairment of bone matrix formation are commonly invoked to explain the osteoporosis of hyperthyroidism. However, Askanazy and Rutishauser[11] and Follis[54] have described the histologic appearances of osteitis fibrosa in the bone (Fig. 13), thus showing that there may be an increase in bone destruction. Krane *et al.*[85] have confirmed this by demonstrating that hyperthyroidism is associated with an increase in the turnover of Ca^{45} and have also found that the calcium balance may be negative when the

patient is in positive nitrogen balance. Puppel *et al.*[133] have shown that the oral administration of calcium can produce a positive balance in patients with hyperthyroidism.

These data suggest that the osteoporosis

hypercalciuria is due to a change in renal threshold or to an imperceptible rise in the serum calcium is a matter that requires further investigation; it is probably significant that hypercalcemia has been reported in hyperthyroidism.[83,132,137]

Immobilization

The osteoporosis of immobilization is also attributed commonly to the reduced activity of the osteoblasts in the absence of normal stresses and strains, but the serum alkaline phosphatase, which is supposed to reflect osteoblastic activity, is normal. The development of hypercalcemia in this condition[4] suggests rather that bone is being destroyed than that it is not being formed, and this is the implication of certain other studies. Thus Engström and Amprino[47] have found by autoradiographic technics that the rate of formation of new haversian systems appears to be the same in the immobilized limb of a dog as it is in the control limb, and Armstrong[9] has shown the same in rats. It is interesting that Slack[149] found that there was an increase in the uptake of C^{14}-labeled glycine by the wasting soft tissues of the immobilized limb of the rat.

These data, such as they are, suggest that immobilization is associated with increased destruction of tissues rather than impaired formation Nordin[116] has shown that the solubility of bone powder is closely related to pH, and one wonders if circulatory stasis could not lead to a fall in pH in an immobilized limb and so to removal of calcium.[109]

Cushing's Syndrome

It is quite possible that the osteoporosis of Cushing's syndrome and of cortisone administration[40] is due to interference with the synthesis of bone matrix. Several groups of



FIG. 13. Examples of osteitis indicating active bone destruction in hyperthyroidism. (Follis, R. H., Jr.: Bull. Johns Hopkins Hosp 92:413)

workers have shown that cortisone administration delays the healing of fractured bones in animals,[26,101,148] and it is generally accepted that the glucocorticoids of the adrenal cortex depress protein synthesis. On the other hand, there seems to be definite osteoclastic activity—suggesting bone destruction—in some of the published histologic sections of bone from patients with Cushing's syndrome.[147] Moreover, Wyman and Robbins[167] have reported the absence of the lamina dura of the teeth in patients with Cushing's syndrome. This is a radiologic sign that hitherto has been taken to signify

bone destruction. Further studies are required before this form of osteoporosis can be explained satisfactorily and classified.

Scurvy

There is no reason to suppose that the osteoporosis of scurvy[15] is due to negative calcium balance. On the contrary, the disturbance in ground substance that occurs in this condition suggests that matrix synthesis may be impaired, as Albright and Reifenstein suggest. It may be significant that this is the one form of osteoporosis in which the level of serum alkaline phosphatase, which is believed to reflect osteoblastic activity, is depressed.[127]

SUMMARY AND CONCLUSIONS

The general belief that osteoporosis is a disorder of the protein of bone rests on unsatisfactory evidence. Experimental work, the significance of which has not been sufficiently appreciated, shows that calcium deficiency causes osteoporosis in animals, whereas protein deficiency does not. Dietary surveys show that low calcium intakes are not uncommon in the Western Hemisphere, and balance studies suggest that some subjects are unable to adapt to low calcium diets and so remain, apparently indefinitely, in negative calcium balance. In particular cases, malabsorption of calcium and/or hypercalciuria could lead to negative balance even when the intake of calcium appears to be adequate, and it seems probable that prolonged negative calcium balance, however it is produced, gives rise to the clinical picture of osteoporosis unless sufficiently rapid to cause osteitis fibrosa The preponderance of this condition among women could be explained by the calcium drain of pregnancy and lactation, but there may be an endocrine factor.

Rickets and osteomalacia should be regarded as a single disease, the essential feature of which is the low product of Ca $\times$ P (or $Ca^3 \times P^2$) in serum and consequent failure of new osteoid to calcify. This low serum product is usually the result of a reduction in the level of serum phosphate produced by parathyroid overactivity in response to a reduced level of serum calcium. The fall in serum calcium, in its turn, is due probably to a deficiency of the vitamin D factor that normally sustains the serum calcium by a direct action on bone.

Thus, rickets is characterized by an abnormality in the serum biochemistry; osteoporosis is characterized by a reduction in bone mass. They are distinct entities but may coexist when a patient with vitamin D deficiency and a low product of serum calcium and phosphate is also in negative calcium balance due to malabsorption of calcium.

It is not claimed that all forms of osteoporosis can be explained on the lines presented here, but it is believed that so-called postmenopausal and senile osteoporosis could be accounted for in this way, and that possibly certain other varieties may prove to be the result rather than the cause of negative calcium balance. Preliminary work in support of the views advanced in here have been reported elsewhere.[117]

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Osteomalacia, Osteoporosis e Carentia de Calcium

Summario in Interlingua

Osteoporosis es definite como un reduction del quantitate de osso in le skeleto o in un parte del skeleto, ben que le phenomeno es accompaniate de nulle cognoscite alteration qualitative del osso. Le conception orthodoxe al tempore presente es que iste condition es le effecto de un indeterminate defecto in le proteina de osso.

Il es ben cognoscite que rachitis e osteomalacia es usualmente causate per un carentia de vitamina D. Le reduction del concentracion seral de calcium que resulta ab iste carentia es probabilemente effectuate per le perdita del action calciemic del vitamina super le osso e non per malabsorption de calcium. Illo causa, de su parte, le stimulation del parathyroides e assi un reduction del phosphato seral.

Investigationes in animales, datante de usque a 1872, ha demonstrate uniformemente que le carentia de calcium resulta in osteoporosis plus tosto que in rachitis. Le autor opina que osteoporosis se disveloppa sub iste conditiones proque le carentia de calcium per se non suffice a effectuar un reduction del nivello seral de calcium. Il occurre nulle disturbation del calcification de osso ancian sed un inevitabile destruction de nove osso.

Iste investigationes in animales suggere que osteoporosis clinic—que le autor divide in le typos primari e secundari—es possibilemente le resultato de un prolongate balancia negative de calcium. Un tal balancia negative, de su parte, pote esser causate per carentia dietari de calcium, per malabsorption de calcium, o per excessos del excretion urinari de calcium. Le investigation de casos clinic indica que le majoritate del patientes con osteoporosis primari ha (1) un historia de un basse ingestion de calcium, (2) signos de malabsorption de calcium, o (3) un excessive excretion urinari de calcium.

Le application de iste considerationes fundamental a osteoporosis secundari es apte de revelar le factor etiologic in omne typo particular.

25

Osteopenia in Adolescence

GUNILLA BERGLUND AND BERTIL LINDQUIST*

Rarefaction of bone (osteopenia) is one of the atrophic processes that constitute the picture of senescens. However, in recent years several investigations have been reported disclosing that, although most common in elderly persons, rarefaction of bone without any etiologic factors demonstrable may also occur earlier in life. Below we report two cases, each of an adolescent boy who developed severe osteopenia in the course of about 6 months.

CASE 1

This boy is the second son of healthy parents and has two healthy brothers. Accidentally at 8 years of age he suffered a minimal fracture of the occipital bone; otherwise he has enjoyed good health. He was an excellent gymnast. When he was 12 years old he began to have gradually increasing pains in the dorsal areas of his feet, which slowly curtailed his physical activity.

On admission to the hospital 3 months after the first symptoms he could walk only very short distances with a shuffling gait and complained then of intensive pains in the heels and the foot joints. His general condition was good Routine examination of the internal organs did not reveal anything of interest. A complete neurologic examination showed normal conditions. The x-ray findings were a slight decrease in density of the foot skeleton and the pelvis; other parts of the skeleton were normal (Fig. 1, *left*).

Laboratory Investigations. The blood values were normal: hemoglobin, 13.8 Gm. per cent; WBC, 5,000, with a normal differential count. The acid-base balance in serum was normal. Repeated determinations of the serum levels of calcium, phosphorus, alkaline phosphatases, proteins and lipids showed values within normal limits. The glucose tolerance test was normal. Liver and kidney function tests, including tubular reabsorption of phosphate, were all normal. The urinary excretion of the 17-ketosteroids was normal; so also was the excretion of amino acids Determinations of the urinary excretion of calcium showed normal values. A fat balance study failed to reveal any steatorrhea. Two calcium balance studies performed about 6 months after admission disclosed a negative balance of 0.16 Gm. per day. In a third study 3 months later, calcium intake and output were in balance. According to Bauer, Carlsson and Lindquist,[2,3] a kinetic study with Ca^{47} revealed an accretion rate of 2.1 Gm. calcium per day (table on p. 260 and Fig. 2). A histologic examination of a biopsy specimen from the tibia shaft showed an advanced destruction of the bone tissue, indicating increased bone resorption (Engfeldt).

Course. During the first months of the boy's stay in hospital, physiotherapy brought about a certain improvement in mobility, but still he was incapacitated to a great extent. Another roentgenogram revealed a marked decrease in density throughout the skeleton with compression of many of the vertebrae, especially those in the thoracal columna. In addition to calcium and vitamins, treatment with norethandrolone (Nilevar), in a dose of 20 mg. a day, was started and continued for about 6 months. During this time, generally he felt a little better, though there was no appreciable improvement in mobility. A further roentgenogram of the skeleton showed mainly the same situation as before (Fig. 1, *right*). As of this writing his condition is unchanged.

CASE 2

A previously healthy boy, first child of healthy parents, and with a younger sister in good health. When he was 9 years old he fell

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Investigationes in animales, datante de usque a 1872, ha demonstrate uniformemente que le carentia de calcium resulta in osteoporosis plus tosto que in rachitis. Le autor opina que osteoporosis se disveloppa sub iste conditiones proque le carentia de calcium per se non suffice a effectuar un reduction del nivello seral de calcium. Il occurre nulle disturbation del calcification de osso ancian sed un inevitabile destruction de nove osso.

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Le application de iste considerationes fundamental a osteoporosis secundari es apte de revelar le factor etiologic in omne typo particular.

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Osteopenia in Adolescence

GUNILLA BERGLUND AND BERTIL LINDQUIST*

Rarefaction of bone (osteopenia) is one of the atrophic processes that constitute the picture of senescens. However, in recent years several investigations have been reported disclosing that, although most common in elderly persons, rarefaction of bone without any etiologic factors demonstrable may also occur earlier in life. Below we report two cases, each of an adolescent boy who developed severe osteopenia in the course of about 6 months.

CASE 1

This boy is the second son of healthy parents and has two healthy brothers. Accidentally at 8 years of age he suffered a minimal fracture of the occipital bone: otherwise he has enjoyed good health. He was an excellent gymnast When he was 12 years old he began to have gradually increasing pains in the dorsal areas of his feet, which slowly curtailed his physical activity.

On admission to the hospital 3 months after the first symptoms he could walk only very short distances with a shuffling gait and complained then of intensive pains in the heels and the foot joints His general condition was good Routine examination of the internal organs did not reveal anything of interest. A complete neurologic examination showed normal conditions The x-ray findings were a slight decrease in density of the foot skeleton and the pelvis; other parts of the skeleton were normal (Fig. 1, *left*).

Laboratory Investigations. The blood values were normal: hemoglobin, 13.8 Gm per cent, WBC, 5,000, with a normal differential count. The acid-base balance in serum was normal Repeated determinations of the serum levels of calcium, phosphorus, alkaline phosphatases, proteins and lipids showed values within normal limits. The glucose tolerance test was normal Liver and kidney function tests, including tubular reabsorption of phosphate, were all normal. The urinary excretion of the 17-ketosteroids was normal; so also was the excretion of amino acids. Determinations of the urinary excretion of calcium showed normal values. A fat balance study failed to reveal any steatorrhea. Two calcium balance studies performed about 6 months after admission disclosed a negative balance of 0.16 Gm. per day. In a third study 3 months later, calcium intake and output were in balance. According to Bauer, Carlsson and Lindquist,[2,3] a kinetic study with Ca^{47} revealed an accretion rate of 2.1 Gm. calcium per day (table on p. 260 and Fig. 2). A histologic examination of a biopsy specimen from the tibia shaft showed an advanced destruction of the bone tissue, indicating increased bone resorption (Engfeldt).

Course. During the first months of the boy's stay in hospital, physiotherapy brought about a certain improvement in mobility, but still he was incapacitated to a great extent. Another roentgenogram revealed a marked decrease in density throughout the skeleton with compression of many of the vertebrae, especially those in the thoracal columna In addition to calcium and vitamins, treatment with norethandrolone (Nilevar), in a dose of 20 mg. a day, was started and continued for about 6 months During this time, generally he felt a little better, though there was no appreciable improvement in mobility. A further roentgenogram of the skeleton showed mainly the same situation as before (Fig. 1, *right*). As of this writing his condition is unchanged.

CASE 2

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FIG. 1. Roentgenograms showing vertebral changes in adolescent osteopenia (Case 1). (*Left*) On admission to the hospital. (*Right*) 15 months later

on the ice, after which he had moderate pains in the back for about a week. Six months before admission to the hospital he fell again on the ice and suffered a fracture of the left radius and ulna, which healed with a slight dislocation. A roentgenogram of the arm did not reveal anything abnormal in the bone structure Some months afterward he started to complain of increasing stiffness in his back, especially in the mornings, when he could not straighten up, and of slight pains on sudden movements.

On admission to the hospital he was a well-developed 12-year-old boy, not yet at puberty. He was greatly restricted and moved like an old man; his capacity to bend his back especially was very limited. Routine examinations of internal organs showed nothing of interest

ACCRETION RATE (A) AND EXCHANGEABLE FRACTION (E) OF CALCIUM IN THE ENTIRE BODY AS COMPUTED FROM CA^{47} DATA IN 2 PATIENTS WITH OSTEOPENIA

Subject and Sex	Age (Yrs)	Body Weight (Kg)	Rate Constants* (Fraction/ Day)	Coefficients* (% Dose/ Gm. Ca)	Exchange-able Fraction (E) (Gm. Ca)	Accretion Rate (A) (Gm Ca/Day)	Excretion Rate (U) (Gm. Ca/Day)
1	14	41	0.40	13 5	6.2	2 06	0.38
2	14	58	0.26	8 2	9.0	2.11	0.23

* These values refer to the final exponential on the semilogarithmic plot.

Neurologic examination revealed normal conditions. A roentgenogram showed compression of practically all vertebrae in the thoracal and the lumbar columna below T 4; the height of the vertebrae of T 4, 5, 8 and 10 and L 1, 2 and 5 was reduced to about half of normal (Fig. 3, *left, top and bottom*). There was a marked decrease in density throughout the whole columna. A urography was normal.

Laboratory Investigations. The blood values were normal: hemoglobin, 13.0 Gm. per cent; WBC, 3,500 to 9,800. Total eosinophil count was 427/mm³. Electrolyte studies revealed a normal acid-base balance. Repeated determinations of the serum levels of calcium, phosphorus and alkaline phosphatases showed values within normal limits. The glucose tolerance test was normal. The urinary excretion of 17-ketosteroids and amino acids was normal.

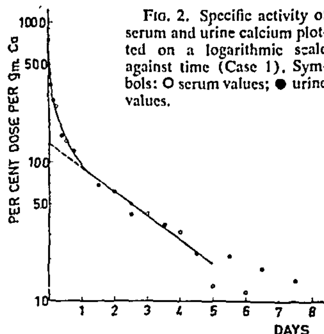


Fig. 2. Specific activity of serum and urine calcium plotted on a logarithmic scale against time (Case 1). Symbols: O serum values; ● urine values.

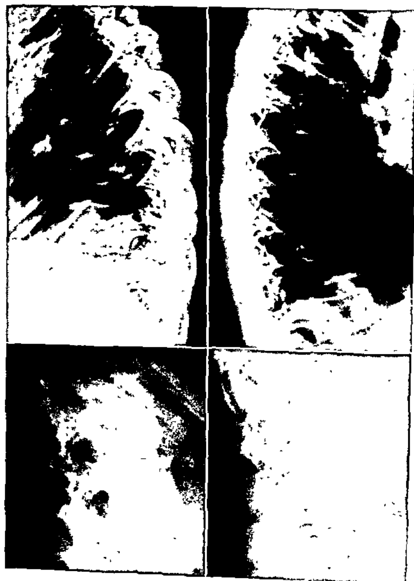


Fig. 3. Roentgenograms showing vertebral changes in adolescent osteopenia (Case 2). (*Left, top and bottom*) On admission to the hospital. (*Right, top and bottom*) 5 months later.

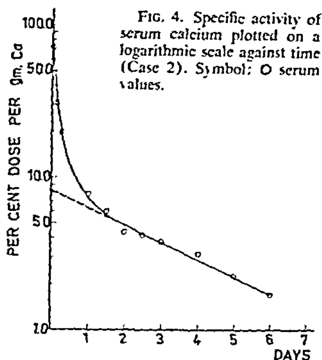


FIG. 4. Specific activity of serum calcium plotted on a logarithmic scale against time (Case 2). Symbol: O serum values.

Liver and kidney function tests, including tubular reabsorption of phosphates, were all normal. Determinations of urinary excretion of calcium revealed normal values. A fat balance study failed to reveal any steatorrhea. In a calcium balance study the intake and the output were in balance. According to Bauer, Carlsson and Lindquist,[2,3] a kinetic study with Ca^{47} showed an accretion rate of 2.1 Gm. calcium per day (table on p. 260 and Fig. 4). In a histologic examination of the spinal process there were signs of intensive metabolism of the bone with dominance of the resorption processes The bone structure seemed to be normal with a fair amount of cementation lines (Engfeldt).

Course. In the first 5 months after admission to the hospital the patient received no other treatment than the application of an orthopaedic corset to support his back. During this time his stiffness increased, and he complained of lumbar pains every morning. Roentgenographically there were increased compressions of the vertebrae but no changes in the density of the vertebrae as compared with the roentgenograms taken 5 months before (Fig. 3, *right, top and bottom*) At this time, therapy was started with nortestosterone (Durabol), 5 mg. intramuscularly every 10th day, and, as of now, the patient has been getting this treatment for 7 months During this time his general condition has improved, and the pains have disappeared. The mobility of the columna has increased; he can bend forward, place his hands just below the knees and straighten up immediately afterward, and he is able to walk distances of 70 odd feet without difficulty. However, the roentgenogram shows practically the same situation as before.

DISCUSSION

In both patients the disease started just before puberty in previously healthy boys. In Case 1 the beginning symptom mainly was pain; in Case 2, it was an increasing immobility. The roentgenogram as well as histology, showed in both cases an abnormal situation, indicating a reduction of the bone mass in the skeleton.

A quantitative reduction of bone mass without a qualitative change in its composition often is termed *osteoporosis*. This term is used also in the strict sense of Albright and Reifenstein;[1] i.e., exclusively for conditions of rarefaction of bone supposed to be caused by a decreased rate of bone formation, in turn secondary to a deficient matrix formation. The term *osteopenia* has been introduced by Bauer, Carlsson and Lindquist[4] to cover conditions of rarefaction of bone in the broader sense. Thus osteopenia is equivalent to conditions of "too little calcified bone" in the terminology used by Albright and Reifenstein.[1,5]

The most common cause of osteopenia in adolescence is probably Cushing's disease. Often it starts in the columna, to which it may be restricted, though it also may be generalized. However, this diagnosis is ruled out by the fairly long follow-up of the patients here reported—about 2 years—without any other symptoms arising of morbus Cushing, in addition to the normal urinary excretion of 17-ketosteroids and the normal glucose tolerance test A hyperparathyroidism, primary or secondary, may also give rise to a generalized decalcification of the skeleton, as observed in the present cases. The normal serum calcium and serum phosphorus levels, besides the normal tubular reabsorption of phosphate, make this diagnosis improbable. Furthermore, disuse can hardly be regarded as the cause of the osteopenia because of the fact that during

the year prior to the onset of symptoms both patients were physically active. Nor has it been possible to reveal any other etiologic factors in these patients.

In metabolic bone disease certain investigations of mineral metabolism usually are performed in order to elucidate the mechanism behind the development of the disorder. In the cases here reported the urinary excretion of calcium was normal; osteopenia usually develops slowly, and, as there is only a slight discrepancy between formation and resorption of bone, the hypercalciuria often is not marked and may not be evident. Balance studies reveal if rarefaction of bone still is taking place, if the reverse prevails, or if the processes of bone formation and resorption compensate each other. In this respect the balance studies performed gave informations as to the actual phase of the disease. The kinetic analysis reveals the rate of skeletal turnover at which the osteopenia develops.

Osteopenia is the result of retarded bone formation and/or accelerated bone resorption. By tracer studies it is possible to determine at what rate of bone salt formation (accretion), such as skeletal disorder develops. Both cases presented here were found to have accretion rates of 2.1 Gm. calcium per day. This value is within the limits found in normal subjects in adolescence.[5,3] Thus, these figures would suggest that in these cases of osteopenia the processes responsible for bone salt accretion were normal. However, in view of the presence of multiple fractures in both cases, higher than normal accretion values might perhaps have been expected. Therefore, the possibility that the osteopenia had developed at a subnormal rate of bone formation cannot be excluded.

A normal accretion rate in osteopenia of adults 42 to 65 years old has recently been reported by Heany and Whedon.[7] Theoretically, however, osteopenia may develop at a high, a decreased or a normal rate of accretion. In one case of osteopenia in a 50-year-old man a study of the accretion rate in the compact bone of tibia by means of P^{32} revealed a decreased value compared with a control subject of corresponding age and weight.[2a] A decreased, as well as a normal accretion rate in osteopenia has been found by Eisenberger and Gordan.[6]

Judged by clinical findings, the pathophysiologic mechanisms operating in osteopenia probably are multiple. The fact that it has been found that the accretion rate does not change in the same way in all patients with this disorder supports this opinion.

SUMMARY

Two cases of osteopenia in adolescence are reported. In both of them—boys—the disorder started at 12 years of age: in one case with severe pains in the heels and later in the back; in the other with a highly decreased mobility of the back per se. Within 6 months a marked rarefaction of the skeleton, especially the columna, developed with several vertebral compression fractures. In both cases kinetic studies by means of Ca^{47} revealed a normal rate of bone formation.

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Osteopenia in Adolescentes

Summario in Interlingua

Es reportate duo casos de osteopenia in adolescentes. Ambe le patientes es masculos. In ambes le disordine comenciava al etate de 12 annos: in le un con dolores sever in le calce e subsequentemente le dorso e in le altere con un grandemente reducite mobilitate del dorso. Intra 6 menses marcate grados de rarefaction skeletic, specialmente del columna vertebral, se disveloppava e deveniva le causa de plure fracturas de compression in vertebras. In ambe casos, studios cinetic per medio de Ca^{47} revelava un normal intensitate del formation de osso.

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Studies of the Oral Toxicity of Strontium Chloride in Rats*

HAROLD C. HODGE, PH.D., MARGARET W. NEUMAN, PH.D., AND HARVEY J. BLANCHET, JR., M.D.

On January 9 and 10, 1950, at the Second Conference on Metabolic Interrelations, held in New York under the auspices of the Josiah Macy, Jr. Foundation, Dr. Franklin C. McLean presided while Dr. Ephraim Shorr reported studies on the value of strontium as an adjuvant to calcium in the remineralization of the skeleton in osteoporosis in man.[16] For some years Dr. Shorr had explored the possibility that strontium might serve as an equivalent of the normal lime salts for patients with advanced osteoporosis. He showed that in doses of 1.5 to 2.75 Gm. per day of strontium (in conjunction with a high-calcium diet), part was retained in the body, but he stressed the concomitant increase in total alkaline earth retention (Ca + Sr).

Dr. Shorr had no misgivings about the innocuous nature of strontium salts.[9] However, the paucity of data on the acute and the short-term toxicity of large doses of strontium administered orally prompted the limited study reported here.

The *acute* toxicity of parenterally administered strontium as reported in the literature is recorded in the table below.

The *chronic* toxicity has been studied principally by feeding strontium in conjunction with rachitogenic diets;[14,8] for example, at levels of about 2 per cent. The resulting intensification of the rachitic lesion was presumed to be caused by strontium's inhibition

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ACUTE TOXICITY OF STRONTIUM, PARENTERAL ADMINISTRATION
(DATA ASSEMBLED FROM THE LITERATURE)

Compound	Animal	Route of Administration	Toxicity mM./Kg.	References on pp. 267 & 268
Strontium acetate	Rat	I.V.	LD_{50} 1.16	4
Strontium chloride	Rat	I.V.	MLD 1.4	9
Strontium chloride	Rabbit	I.V.	MLD 6.6	5
Strontium chloride	Rabbit	I.V.	MLD 5.0	18
Strontium iodide	Rat	I.P.	LD_{50} 1.77	3
Strontium bromide	Rat	I.P.	LD_{50} 2.80	3
Strontium lactate	Rat	I.P.	LD_{50} 2.80	3
Strontium salicylate	Rat	I.P.	LD_{50} 1.0	3
Strontium nitrate	Rat	I.P.	LD_{50} 2.52	3
Strontium chloride	Mouse	I.P.	LD_{50} 9.0	10

of phosphate absorption. A rachitogenic action has also been observed histologically in bones of rats fed a normal diet containing 2 per cent $SrCl_2$ but not in those fed 1 per cent $SrCl_2$.[1] Even when the amount of strontium fed (0.05 M. strontium lactate in the drinking water of mice) was too low to produce observable rachitic lesions, a reduced calcium content was observed in the bone ash.[10]

This is not the kind of action one would expect from an agent beneficial in osteoporosis. It was a puzzle to Dr. Shorr at this Macy conference, particularly as Sobel[17] discussed the role of strontium as a competitor with calcium for the local calcification mechanism in in vitro cartilage slices. Later, at this conference, Marks and Shorr[11] reported the in vitro deposition of strontium in rachitic cartilage slices but offered no evidence of the stimulation of calcification.

Follis, who showed an interest in the role of strontium in osteoporosis at this conference,[6] subsequently administered strontium to normal rats, starved rats and animals with fractures, in all of which he observed an unusual growth of osteoid, confirming the original observation of Shipley *et al.*[14] Follis suggested that strontium might stimulate matrix formation in osteoporosis.[7]

As it turned out, the initial promise of Dr. Shorr's treatment was not borne out by continued clinical trials.[15] This is not surprising in view of the recent voluminous literature (stimulated by the widespread interest in Sr^{90}) showing conclusively that strontium is not retained in the body nearly as well as is calcium.[12] The discrimination takes place principally at the point of resorption in the kidney in a ratio that is rigidly maintained even with various changes in the serum concentrations of calcium and strontium.[2]

Reported below are the results of studies of rats given orally relatively large doses of $SrCl_2$ either by stomach tube or admixed in an otherwise normal diet for a period of about 1 month.

EXPERIMENTAL

Acute Oral Toxicity

To determine the acute oral toxicity, 0.75 M. $SrCl_2$, pH 5.7, was administered by stomach tube to 170 female Rochester strain (ex-Wistar, 1923) albino rats weighing between 160 and 230 Gm.; average weight 183 Gm. Before intubation, the animals were fasted 24 hours and after that were given food *ad lib.* for an observation period of at least 5 days.

Shortly after administration of lethal doses, the rats evidenced excessive salivation, rhinorrhea, diarrhea and restlessness; later, motor depression. Most deaths occurred within 24 hours after administration, a few in the second and the third days, and only one as late as 5 days. The LD_{50}, as determined graphically, was 14.1 ± 0.5 mM./Kg. The cause of the death appeared to be respiratory failure. Cursory autopsies revealed no gross abnormalities other than lung hemorrhage.

As an indication that the chloride was not significantly responsible for the mortality, equivalent doses of NaCl were given to 15 rats and proved to be nonlethal. At higher doses, 1 animal survived 60 mM./Kg. and 1 succumbed to 120 mM Kg. of NaCl.

Preliminary tests involving only a small number of rats indicated that the toxicity of $Sr(NO_3)_2$, given by stomach tube, was roughly equivalent to that of $SrCl_2$.

Short-Term Feeding Tests

Groups of 15 male and 15 female weanling albino rats with equal litter-mate distribution were maintained for about 1 month on a ration of fox chow meal with meat (Purina) containing 0, 0.5, 2.0 or 20 per cent $SrCl_2$. The high mortality response to 20 per cent $SrCl_2$ prompted further experimentation. Three groups of 10 males and

10 females were given diets containing 0, 5 and 10 per cent $SrCl_2$, respectively. In all cases, food was given *ad lib.* The animals were weighed at least once a week. After 1 month, all animals were sacrificed, organ weights were recorded, and tissue samples were taken for microscopic examination.

Of the 70 controls, 1 male rat succumbed. Two of the males receiving 0.5 per cent $SrCl_2$ also died. In the group receiving 20 per cent $SrCl_2$, all animals were dead by the third week of the experiment.

The rats given diets containing 0.5 per cent $SrCl_2$ grew approximately as well as did the control rats. All the remaining strontium-fed rats evidenced a depression in growth rate that increased with the dose. At levels of 2 per cent, this growth inhibition was of doubtful significance, with the possible exception of the male group fed 2 per cent $SrCl_2$.

The liver, kidney, lungs, heart, brain, spleen, stomach and testes were weighed at the time of sacrifice. No data from the animals receiving 20 per cent $SrCl_2$ are included because of excessive tissue autolysis. There was a small decrease in all organ weights more pronounced with increasing dose of strontium. However, when the results were expressed as organ weight basis body weight, it was evident that organ growth was inhibited to a lesser degree than was carcass growth. The percentage of body weight devoted to brain, spleen and stomach especially showed marked increases after feeding strontium. These trends reflected the general debilitation of the animals and cannot be interpreted as evidence of any specific toxic effect.

The tissues sampled and prepared (H and E stains) for histologic study were heart, lung, spleen, stomach, large and small intestine, liver, kidney, brain, bone, testes and, in some cases, bladder. No consistent abnormality was observed in organs other than bone. Histologically, the metaphysial structures were not markedly abnormal in rats fed a 2 per cent $SrCl_2$ diet, although more osteoid than normal was present.[13]

Radiographs of the hind legs of 2 male and 2 female rats from each of the treated and control groups were made at termination by the method of Morgareidge.[12] Rickets was severe in the 20 per cent group, marked in the 10 per cent group, just detectable in the 5 per cent group, and absent or undetectable in the 2 and the 0.5 per cent groups.

CONCLUSION

In rats, orally administered $SrCl_2$ appears to be relatively nontoxic.

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Studios del Toxicitate de Chloruro de Strontium Administrate per Via Oral a Rattos

Summario in Interlingua

In januario 1950, in le Secunde Conferentia Super le Interrelationes Metabolic organisate per le Fundation Josiah Macy Jr., Dr. Franklin C. McLean presideva durante que Dr. Ephraim Shorr reportava su experientias in le administration de strontium a patientes human suffrente con osteoporosis. Dr. Shorr acceptava le innocentia de sales de strontium. Nonobstante, le paucitate del datos disponibile relative al acute toxicitate a curte vista de grande doses de strontium in compositos administrate per via oral suggereva le desirabilitate de studios additional. Le presente investigation in rattos esseva stimulate per ille reflexiones. Le dose letal median de chloruro de strontium in rattos albin esseva estimate a 14 mM. per Kg. Gruppos de 15 rattos mascule e de 15 rattos feminin esseva mantenite durante periodos de circa un mense con dietas continente 0, 0,5, 2, e 20 pro cento de chloruro de strontium. Gruppos additional de 10 rattos mascule e de 10 rattos feminin recipeva dietas con 0, 5, e 10 pro cento de chloruro de strontium. Multe rattos recipiente le dietas a 20 pro cento moriva. Le rattos con dietas a 0,5 pro cento cresceva normalmente. Plus alte procentages deprimeva le crescentia. Alterationes in le pesos del organos reflecteva le debilitate general del animales e non specific effectos toxic. Meticulose studios histologic revelava nulle uniforme anormalitate excepte in le ossos. Rachitis esseva absente o non detegibile in le gruppos recipiente dietas a 0,5 e a 2 pro cento, justo detegibile in le gruppos con dietas a 5 pro cento, e marcate o sever in le gruppos con dietas a plus alte procentages de chloruro de strontium.

27

Radioisotope Studies of Generalized Skeletal Disorders*

Vitamin D Resistant Rickets

William Meltzer, M.D., Irving Lyon, Ph.D., Esther D. Mensen, M.A., and Robert D. Ray, M.D., Ph.D.†

INTRODUCTION

The purpose of this chapter is to review briefly the use of bone-seeking radioactive isotopes in studying generalized skeletal disorders and to illustrate the principles involved by presenting 2 cases of vitamin-D-resistant rickets and 1 normal patient studied with Sr^{85}.

Our current concepts of bone metabolism may be said to date from the studies of Belchier and Duhamel who found, using madder, silver rings placed round the shafts of long bones and markers drilled into the cortices, that bone growth was appositional rather than interstitial and that the deposition of new bone matrix was accompanied simultaneously by resorption of old. Even in the adult, in whom the total mass of the skeleton is constant, bone formation and resorption are occurring continuously. The next major advance in our knowledge of bone metabolism came with microscopic studies of bone and the description of osteoblasts by John Goodsir, osteoid by Virchow, and osteoclasts by Kölliker, followed by attempts to correlate the histologic picture of the cells and intercellular matrix of bone with its physiologic activity: the number of osteoblasts and the appearance of the cement lines with the rate of bone formation, the number of the osteoclasts and the size of the haversian systems and lacunae with bone resorption, and the amount of osteoid with the rapidity of bone formation and disorders of calcification. Morphologic studies of the skeleton were followed by attempts to correlate changes in serum electrolytes with derangements of skeletal metabolism. It soon became apparent that although the serum levels of calcium in particular, and also of phosphorus, tended to remain constant, alterations in their levels could be related to alterations in skeletal metabolism. Robison's discovery of serum alkaline phosphatase in 1923 permitted further extension of these studies. With the turn of the century the discovery of roentgen rays provided still another technic for studying bone metabolism; in essence, an extension of morphologic studies to changes in shape and density of the skeleton. However, accurate quantitative measurements of alterations in roentgen density have only recently been possible (Keane *et al.*).[4] The next major step in our knowledge of bone metabolism came with the balanced nutritional studies of Osborne, Mendel, Sherman, Dubois, McCollum, Hess, Aub, Albright and others. By means of these studies the relation between

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* These studies were carried out under A.E.C. Contract No AT(11-1)—507.

† The Department of Orthopaedics, Presbyterian-St Luke's Hospital and the University of Illinois College of Medicine, Chicago, Ill.

absorption and excretion of nitrogen, calcium and phosphorus and generalized disorders of the skeleton was further defined. Such studies are still used extensively, and their limitations, as well as their advantages, should be recognized clearly. Basically, a balance study gives the net difference between two processes; for example, between bone formation and resorption. It is impossible by balance studies to estimate the actual rate of bone formation or resorption, and conclusions with regard to alterations in rates of skeletal anabolism or catabolism must be drawn by inference rather than direct evidence. Also, balance studies require large staffs and long-term hospitalization, and they involve considerable expense.

With the advent of radioactive tracers, new technics became available for studying physiologic processes. One of the first clinical applications was the measurement of physiologic "volumes" or "spaces." The principle involved is an old one—that of dilution. A known amount of isotope is injected into an unknown volume, and the concentration is determined subsequently. To simplify the method, it is usually assumed (1) that there is a finite mixing or equilibration time, (2) that at equilibrium the isotope is distributed uniformly throughout the volume, (3) that after equilibration is reached there is no appreciable loss of isotope from the system, and (4) that the volume of the system remains constant during the interval under study. Granting these assumptions, the volume may be determined by the following relationship:

$$\text{Volume of distribution} = \frac{\text{Amount of isotope injected}}{\text{Final concentration of isotope}}$$

A second application of radioactive tracers to physiologic problems was the measurement of changes in rates of flow, growth or decay. The technic involved here may be illustrated diagrammatically below in Figure 1.

Following introduction of a tracer into such a system, there will be a gradual decrease in its concentration, as it is both lost and diluted. To determine the volume of the system and the rate of loss of the isotope and its stable homologue, several new assumptions must be made: (1) the rate of flow must be constant (i.e., the system must be in a steady state); (2) the mixing or equilibration time should be relatively short compared with the time interval in which the rate of loss from the system is calculated; and (3) there must be no return or feedback of tracer during the interval

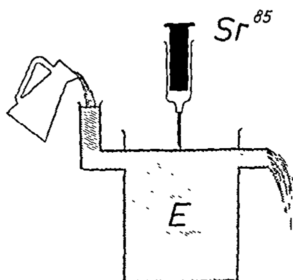


FIG. 1. See text for discussion.

under study. If these assumptions are valid, the concentration of isotope in the system at any time may be described by the following relationship:

$$C_t = C_0 e^{-kt}$$

where C_t is the concentration at time t, C_0 the theoretic (effective) concentration at zero time (obtained by extrapolating the curve of concentration, plotted semilogarithmically against time, to zero time), and "k" the fractional removal rate. The rate constant "k" may be determined from the half-life of the tracer in the system as follows:

$$k = \frac{\ln 2}{t_{1/2}} = \frac{0.693}{t_{1/2}}$$

where $t_{1/2}$ is the amount of time required for the removal of half of the injected tracer and presumably the time required for removal of half of the unlabeled homologue. For a complete description of the principles involved, the reader is referred to Siri.[6]

It would appear in the case of bone salt metabolism that one is dealing with a situation approximating the theoretic model described above, except that there are two routes of loss of isotope (excretion and skeletal accretion) rather than one. Following intravenous administration of a radioactive bone-seeking isotope (such as Ca^{45}, Ca^{47}, Sr^{85} or Sr^{90}), there is a rapid drop in plasma concentration. Three simultaneous processes appear to account for this: (1) diffusion of isotope from the blood stream into the body fluids and into bone with establishment of a steady state between the body fluids and that portion of the bone salt in exchange with the body fluids;* (2) loss from the blood stream and body fluids by way of urinary and fecal excretion; and (3) loss of the isotope by incorporation (accretion) into the nonexchangeable fraction of the bone salt. Once mixing and equilibration have occurred, the subsequent fall in plasma concentration is due to the last two processes alone. At a later interval the bone in which the isotope was incorporated by accretion is resorbed or remodeled, and isotope previously deposited is mobilized and recirculated. At this point a break in the plasma concentration curve occurs due to re-entry of isotope into the circulation. However, during the interval between the initial establishment of a steady state and recirculation of the isotope, the conditions outlined for the theoretic model are approximated, and the plasma concentration curve approximates a straight line when plotted semilogarithmically against time.

In clinical studies, the amount of isotope lost from the body can be determined by direct measurement of the urine and the feces. (An exception must be made for pregnancy and lactation.) The isotope remaining in the body may be considered as partitioned between two fractions: one fraction in the exchangeable pool and a second fraction lost from the pool by skeletal accretion. Two unknowns remain: the volume of the exchangeable space and the rate of loss of isotope by skeletal accretion.

The details of the various methods proposed for solving these two unknowns have been presented previously by Bauer, Carlsson and Lindquist,[1] Bauer and Ray,[2] and Ray and Lyon.[5]

METHOD

Carrier-free Sr^{85}, 10 to 20 μc. in 2 to 3 cc. saline, is injected into the antecubital vein of the patient. A plasma activity curve is developed by taking blood samples every 12 hours for 7 days. The heparinized plasma is placed in 2-cc. ampules and counted with a 2-inch sodium iodide scintillation crystal in a well counter. By direct com-

* From the [illegible]

bone crystals in contact with the body fluids. This portion of the bone salt has been termed the *exchangeable fraction*. Thus, the distribution space of a bone-seeking radioactive tracer, such as calcium or strontium, includes not only the extravascular fluid space (cerebrospinal fluid, interstitial fluid, saliva, joint fluid, etc.) but also a solid phase, the *exchangeable fraction* of the bone salt. The combined fluid and solid spaces in which rapid interchange occurs is termed the *exchangeable space*.

parison with a standard, the concentration of isotope in the plasma samples may be determined. *The standard is prepared by* diluting a measured amount of Sr^{85} in a known volume of 1 M $SrCl_2$. A 2-cc. aliquot of this solution is counted under the same conditions as the plasma samples. The plasma content of Sr^{85} as a percentage of the injected dose is determined from the plasma concentration by multiplying the latter by the plasma volume. Twenty-four-hour urine samples are collected and counted in 1,000-cc. plastic bottles. The stools for 4-day periods are homogenized and counted in 2,000-cc. glass bottles. A lagtime of 12 hours is allowed for the stool to pass from the intestine to the rectum.

CASE PRESENTATIONS

Control

History. L. K., a 16-year-old white female, who had had tuberculosis of the left hip at 5 years of age. The patient was left with 3 inches of actual left-leg shortening and a flexion-adduction contracture of that hip. She was fully ambulatory but walked with a marked limp due to the short leg and the contractures

Physical Examination. Height, 5 feet 1 inch; weight, 121 lbs. The patient was normal in all respects except for the left lower extremity. *The left hip was fused clinically in 30° of* adduction and 30° of flexion.

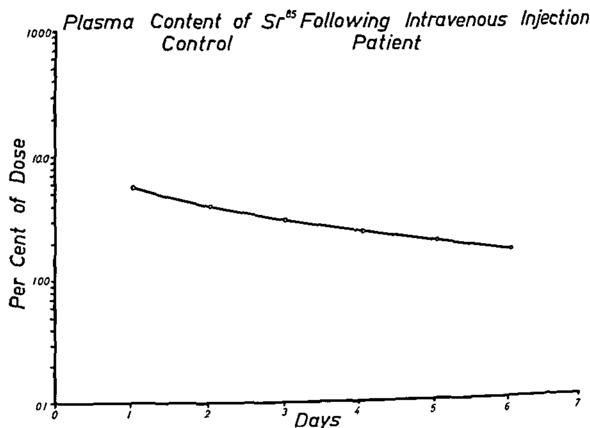
Laboratory Data. The blood counts and the erythrocyte sedimentation rate were normal

X-ray Findings. There was no evidence of active tuberculosis of the lungs or the skeletal *system. Roentgenograms of the left hip re*vealed bony fusion of that joint.

Isotope Studies. The patient was injected intravenously with 15 μc. of carrier-free Sr^{85}. Plasma samples were taken every 12 hours for 7 days Urine and stools were collected for *determination of Sr^{85} content. The results* of these studies are presented in Figures 2 to 5

Patient A

History. K. R., a 19-year-old white female, whose history was progressive bowing of the



Figs. 2 to 5, *Control.* Fig. 2. Note that the plasma concentration of Sr^{85} is plotted semilogarithmically against time. Therefore, theoretically, the concentration will never reach zero. The curves of Figures 10, 14 and 24 are similar to this except for the slopes

Cumulative Excretion (Urinary and Fecal) of Sr^{85}
Following Intravenous Injection
Control - Patient

Per Cent of Dose

Days

FIG. 3. The curves of Figures 3, 11, 15 and 25 are of cumulative excretion in per cent of dose plotted against time. When the daily increments of excretion are plotted against time, a curve is obtained that is parallel to the respective plasma curve.

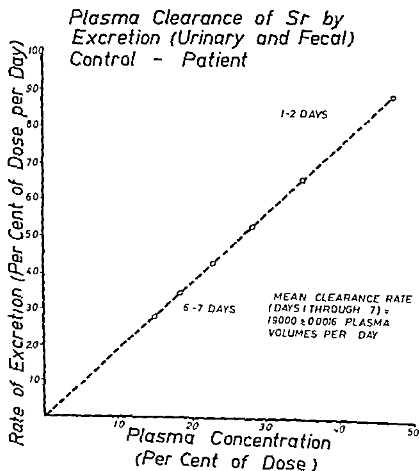


FIG. 4. Figures 4, 12, 16 and 26 illustrate the rate of excretion (per cent of dose per day) plotted against the plasma concentration (per cent of dose per plasma volume). The slope of this curve is the plasma clearance rate of the isotope (plasma volumes cleared of Sr^{85} per day).

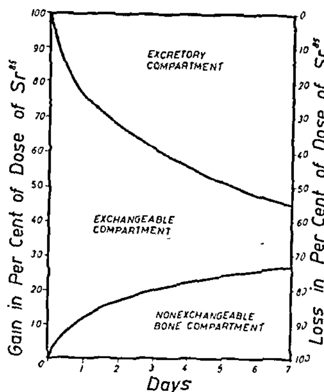
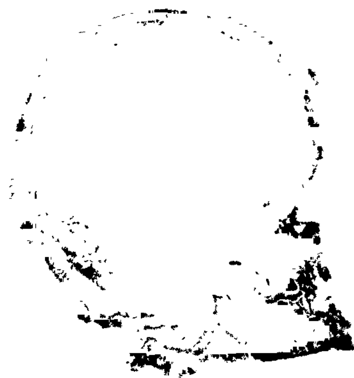


FIG 5. Injected Sr^{85} is distributed among 3 "compartments": excretion, exchangeable (see text and footnote on p. 271) and bone accretion. Figures 5, 13, 17 and 27 illustrate the relative size of each plotted versus time.

extremities and scoliosis since she was 9 years old. Multiple osteotomies, performed in Poland, to correct long-bone deformities had all been followed by recurrence of the deformities. The patient was referred to the Research and Educational Hospital for diagnostic study and therapy. There was no familial history of skeletal disorders.

Physical Examination. Height, 4 feet 1 inch; weight, 92 lbs. The patient was severely retarded in growth and exhibited bilateral frontal bossing; poor dentition; severe, rigid, right dorsal left lumbar scoliosis; marked femoral and tibial bowing; and reversal of the carrying angle of the elbows with dislocation of the heads of both radii. She was able to walk in a walker. The neurologic examination and the remainder of the general physical examination were within normal limits.

Laboratory Data. The hematocrit and the blood counts were normal. Erythrocyte sedimentation rate was 8 mm. per hour. Wassermann and Kahn tests were negative Serum albumin, globulin, electrolytes, calcium and phosphorus were normal The alkaline phosphatase varied between 13 and 15 Bodansky units on several determinations. Intravenous pyelogram and blood urea nitrogen were normal Phenolsulfonphthalein excretion was 25 per cent in 15 minutes. Twenty-four-hour urinary calcium excretion was persistently below 50 mg Twenty-four-hour alpha-amino nitro-



FIGS 6 to 19, Patient A. FIG. 6. Note the marked frontal "bossing"



Fig. 7. The patient had had multiple osteotomies elsewhere, with recurrence of the deformities as shown.



Fig. 8. The marked diminution in radiodensity of the skeleton and advanced vertebral and pelvic deformities are shown. The intravenous pyelogram is normal.

gen was 250 to 500 mg.—the normal is 150 to 230 mg. in 24 hours.* The tubular reabsorption of phosphate was normal.*

X-ray Findings. Roentgenograms revealed the deformities described as well as generalized decrease in osseous radiodensity (Figs. 6-9).

Hospital Course. Preliminary studies with Sr^{85} were carried out, and the results are presented in Figures 10 to 13. The patient then was placed on oral vitamin D, 5,000 units daily. Four weeks later the 24-hour urinary calcium excretion was normal (100-150 mg. per day), and the studies were repeated. The results are presented in Figures 14 to 17.

Histologic Studies. The histologic appearance of the bone is illustrated in Figures 18 and 19.

Patient B

History. L.Z., a 16-year-old white male, was first noted to have bowing of the legs at 3 years of age. At age 6, the diagnosis of vitamin-D-resistant rickets was established on the basis of characteristic roentgenographic changes, decreased serum inorganic phosphorus, elevated serum alkaline phosphatase, and refractoriness to vitamin D therapy except in massive doses. The patient was first seen at this hospital in October, 1957. At that time serum calcium,

* Carried out by the Department of Endocrinology, Presbyterian-St. Luke's Hospital, Chicago, Ill.



Fig. 9. Note the widened shafts of the long and the short tubular bones, narrowed cortices and reversal of the normal carrying angle of the elbow.

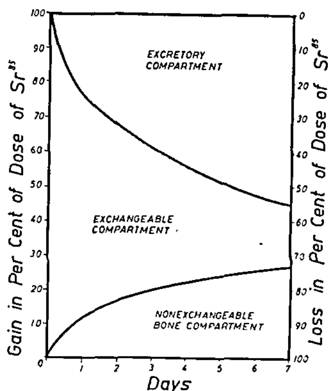


FIG. 5. Injected Sr^{85} is distributed among 3 "compartments": excretion, exchangeable (see text and footnote on p. 271) and bone accretion. Figures 5, 13, 17 and 27 illustrate the relative size of each plotted versus time.

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Physical Examination. Height, 4 feet 1 inch; weight, 92 lbs. The patient was severely retarded in growth and exhibited bilateral frontal bossing; poor dentition; severe, rigid, right dorsal left lumbar scoliosis; marked femoral and tibial bowing; and reversal of the carrying angle of the elbows with dislocation of the heads of both radii. She was able to walk in a walker. The neurologic examination and the remainder of the general physical examination were within normal limits.

Laboratory Data. The hematocrit and the blood counts were normal. Erythrocyte sedimentation rate was 8 mm. per hour. Wassermann and Kahn tests were negative. Serum albumin, globulin, electrolytes, calcium and phosphorus were normal The alkaline phosphatase varied between 13 and 15 Bodansky units on several determinations Intravenous pyelogram and blood urea nitrogen were normal. Phenolsulfonphthalein excretion was 25 per cent in 15 minutes Twenty-four-hour urinary calcium excretion was persistently below 50 mg. Twenty-four-hour alpha-amino nitro-

FIGS 6 to 19, Patient A FIG. Note the marked frontal "bossing."



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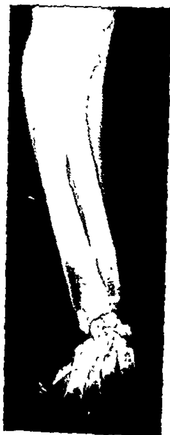


FIG. 9. Note the widened shafts of the long and the short tubular bones, narrowed cortices and reversal of the normal carrying angle of the elbow.

* Carried out by the Department of Endocrinology, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Plasma Content of Sr^{85} Following Intravenous Injection
Vitamin D Resistant Rickets –Patient A
Pretreatment

1000
100
10
01

Per Cent of Dose

0 1 2 3 4 5 6 7
Days

FIG. 10. See caption for Figure 2.

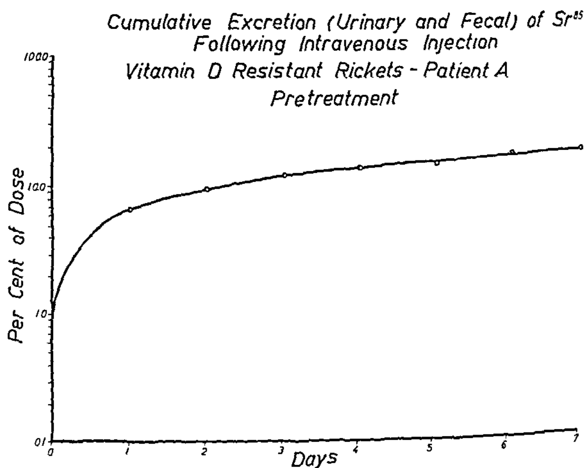


FIG 11. See caption for Figure 3

phosphorus and alkaline phosphatase were normal. He underwent bilateral femoral osteotomies and was discharged on 150,000 units of vitamin D per day. He was readmitted for removal of the intramedullary fixation and metabolic studies.

Physical Examination. Height, 4 feet 9 inches; weight, 98¼ lbs. The patient was short and exhibited moderate femoral bowing. His head was large, but there was no rachitic rosary or Harrison grooves. The upper extremities were normal.

Laboratory Data. Blood studies were normal. Urinary 24-hour calcium excretion averaged 112 mg. (9 determinations). Serum alkaline phosphatase was normal.

X-ray Findings (Figs. 20-22). The preoperative bowing of the femurs is evident, and the method of correction is shown.

Hospital Course. The intramedullary nails were removed from the femurs.

Histologic Studies. The histologic appearance of the bone is illustrated in Figure 23, a biopsy of cancellous bone from the shaft of the femur taken at the time of the first operation.

Isotope Studies. The course of the study was similar to that for the control patient. The results are presented in Figures 24 to 27.

DISCUSSION

The graphs of the isotope studies are self-explanatory, but a few comments may be apropos. Figures 2, 10, 14 and 24 show the interval of time during which the rate of loss of isotope from the plasma is constant. Extrapolation of this part of the plasma curve, expressed as percentage of injected dose per plasma volume, to the ordinate at time zero should give the theoretic concentration of isotope at the instant of injection (P_{t_0}*), thereby correcting for mix-

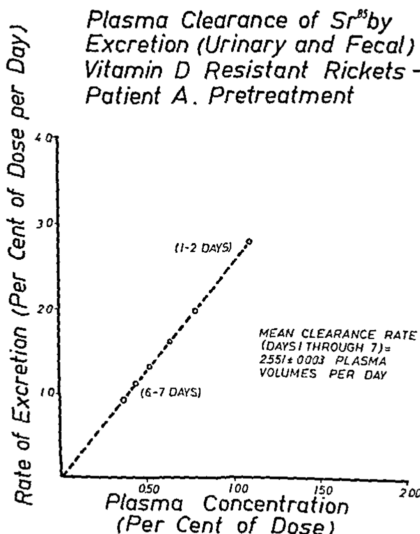


FIG. 12. See caption for Figure 4.

CALCULATED VALUES FOR THE VOLUME (E) OF THE EXCHANGEABLE COMPARTMENT AND THE RATES OF CLEARANCE BY EXCRETION (u) AND BY "ACCRETION" (a)

Patient	Treatment	Exchangeable Fraction Volume of Compartment (Plasma Volumes)	Excreted Fraction Clearance Rate (Plasma Volumes per Day)	"Accreted" Fraction Clearance Rate (Plasma Volumes per Day)
Control	None	17.02	3.24	2.13
Vitamin D Resistant Rickets (A)	None	63.93	3.32	11.73
Vitamin D Resistant Rickets (A)	Vitamin D	56.68	3.53	8.58
Vitamin D Resistant Rickets (B)	Vitamin D	23.94	1.50	5.34

This table lists the values obtained by the analysis described in the text.

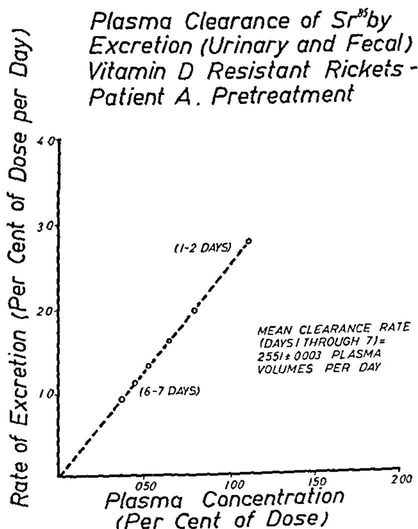


FIG. 16 See caption for Figure 4.

If one plots the *rate of excretion* (percentage of dose excreted per unit time) against the plasma concentration for the corresponding interval (Figs. 4, 12, 16 & 26), the excretory clearance rate of the isotope (u) may be determined from the slope of the line. By taking two different times on the plasma curve and the figure for the excretory clearance rate, one can calculate the accretion rate (a) (expressed as a clearance rate) by simultaneous equations, or by one equation derived from the simultaneous equations:

$$a = \frac{P_{t_1}{}^* R_{t_2}{}^* - P_{t_2}{}^* R_{t_1}{}^*}{P_{t_1}{}^*\left(\frac{U_{t_2}{}^*}{u}\right) - P_{t_2}{}^*\left(\frac{U_{t_1}{}^*}{u}\right)}$$

where $R_t{}^*$ is the amount of isotope retained in the body at time t. ($R_t{}^*$ = injected dose — $U_t{}^*$.) The size of the exchangeable compartment can also be determined by substituting the calculated value for a in the following equation:

$$E = \frac{R_{t_1}{}^* - a\left(\frac{U_{t_1}{}^*}{u}\right)}{P_{t_1}{}^*}$$

The preceding method was presented originally by Bauer and co-workers,[1] and later applied by Bauer and Ray[2] and Ray and Lyon.[5]

Figures 5, 13, 17 and 27 show the changes in the distribution of injected iso-

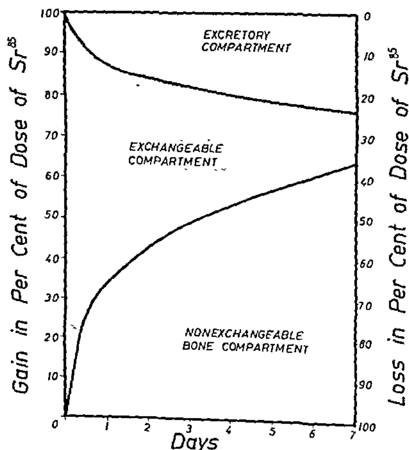


FIG. 17. See captions for Figures 5 and 13.

FIG. 18. Histologic section of cortical bone of the tibia (×530) to illustrate the diameter of a haversian canal and the osteoid border.



FIG 19 Histologic section of trabecular bone of the tibia (×530) to illustrate the large number of osteoblasts and increased width of the osteoid border.

Figs. 20 to 27, Patient B. Fig. 20. No current activity of rickets is present.







Fig. 21 (*Left*). Note the marked bowing deformities of the femurs.

Fig. 22 (*Right*). Note the surgical correction of the deformity. The intramedullary nail has been removed and the correction maintained.



tope for several days in the excreta, exchangeable compartment, and "accreted" fraction of the bone salt. These diagrams were derived by applying the preceding calculations at 24-hour intervals. The table on page 280 summarizes the values for the volume of the exchangeable compartment (E) and the rates of clearance by excretion (u) and by "accretion" (a).

It is not the purpose here to present conclusions with regard to specific disease entities or to review the problem of vitamin-D-resistant rickets[7] but, rather, to illustrate the fact that one can measure alterations in skeletal metabolism by means of parenterally

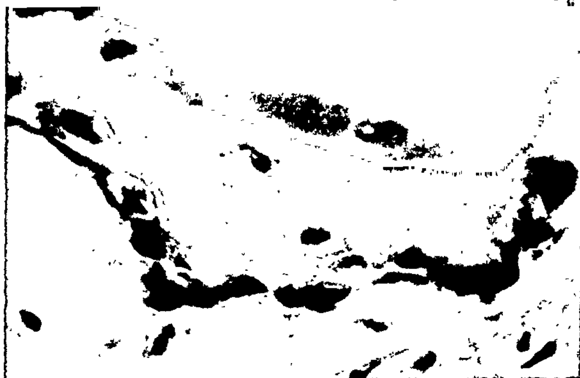


Fig. 23. Histologic section of trabecular bone of the femur (×530) to illustrate the large number of osteoblasts and increased width of the osteoid border.

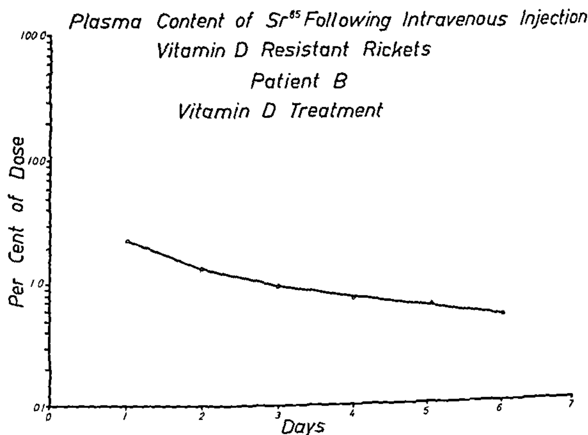


Fig. 24. See caption for Figure 2

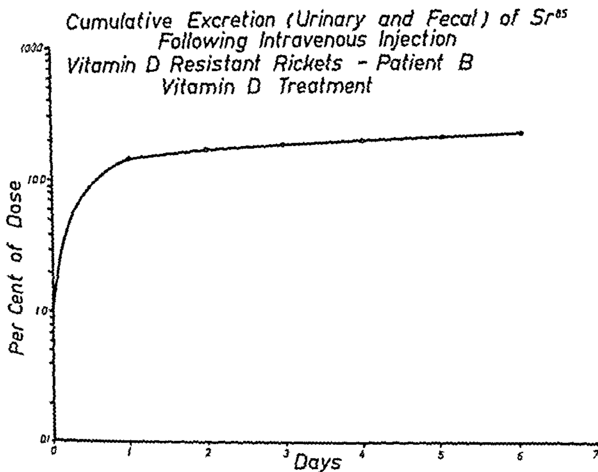


FIG. 25. See caption for Figure 3.

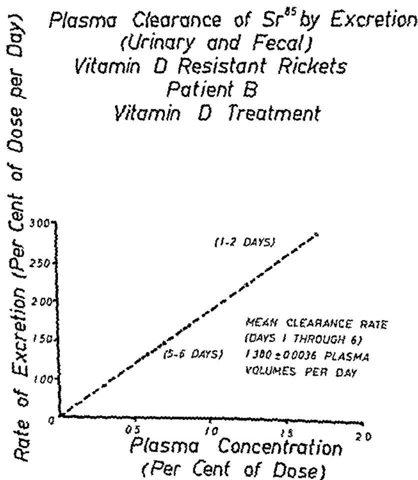


FIG. 26. See caption for Figure 4.

administered bone-seeking radioactive isotopes. Current studies indicate that many skeletal disorders are associated with characteristic alterations in the volume of the exchangeable compartment and the rate of accretion. The two patients with vitamin-D-resistant rickets presented in this chapter exhibited a rapid rate of clearance by accretion when compared with control patients. The rate decreased after administration of vitamin D. Patient B, before vitamin D therapy, exhibited the typical changes of vitamin-D-resistant rickets, including characteristic radiographic findings, bony deformities, decreased serum phosphorus, increased serum alkaline phosphatase, l urinary calcium excretion rate and refr toriness to vitamin D except in mass doses. Patient A is, at best, an atypi example of this syndrome, presenting w severe bony deformities, elevated serum al line phosphatase and low urinary calci excretion rate but normal 24-hour urine c cium on a daily intake of only 5,000 ur of vitamin D.

Differences in the values for the cal lated parameters are presented. The sign cance of these differences as related to cli cal diagnosis and therapeutic evaluation now being investigated.

Distribution of Sr^{85}
Vitamin D Resistant Rickets
Patient B
Vitamin D Treatment

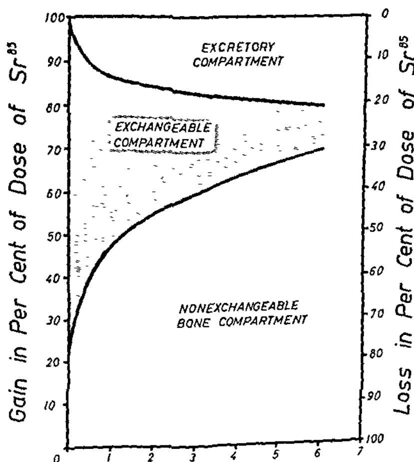


FIG. 27. See captions for Figures 5 and 13.

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Radioisotopos in le Studio de Generalisate Disordines Skeletic; Rachitis Resistente a Vitamina D

Summario in Interlingua

Es presentate un revista historic del progressos effectuate in le studio del metabolismo de osso, culminante in le utilisation de osteotropic isotopos radioactive. Iste ultime include le analyse de specimens serial de plasma, urina, e fece post le administration intravenose de un osteotropic radioisotopo, como per exemplo Sr^{85} sin vector. Studios es presentate que esseva effectuate in duo patientes con rachitis resistente a vitamina D e in un subjecto de controlo. Un del duo patientes con rachitis esseva studiate tanto ante como etiam durante le therapia a vitamina D. Le methodo usate in le analyse del datos es discutite. Es presentate graphicos que illustra le reduction exponential del activitate in plasma in le curso del tempore, le augmento cumulative del activitate in le excrementos, le intensitate del clearance excretori del isotopo (obtenite per registrar le intensitate del excretion como function del concentration in plasma), e le distribution "compartimental" del isotopo in le curso del tempore. Es discutite le notion de un "excambiabile compartimento" pro strontium, e valores numeric es presentate pro le intensitate del accretion skeletic. Le resultatos obtenite in le studio de controlo es comparate con illos observate in le patientes con rachitis resistente a vitamina D. Le signification del differentias es currentemente sub investigation in relation al diagnose clinic e al evaluation del therpia usate.

28

Regulation of Blood Calcium*

D. HAROLD COPP, M.D., ESTHER D. MENSEN, M.A., AND G. DUNCAN MCPHERSON, M.D.†

The level of calcium in blood has been aptly described by McLean and Hastings[23] as one of the critical "physiological constants." Although over 99 per cent of the body calcium is present in the skeleton, the soft-tissue calcium has many important functions, including effects on certain enzyme activities, membrane permeability and neuromuscular excitability. Low levels of calcium ions in tissue fluid may produce tetany and death; high levels produce intestinal and cardiac disturbances, and may cause serious kidney damage. It is not surprising that this is one of the most rigidly controlled constituents of extracellular fluid The acute homeostasis and regulation of blood calcium have been discussed recently in a number of reviews.[9,15,16,17] The more important factors involved are shown diagrammatically in Figure 1. The actual level of calcium in plasma must depend on a balance between calcium added to blood from intestinal absorption and bone resorption and that lost from blood by excretion in the feces, urine and sweat and by deposition in

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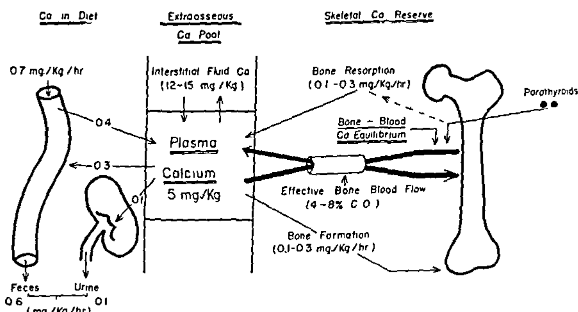


FIG. 1. Diagram of factors affecting plasma calcium. Pool sizes are expressed as mg. calcium per Kg., while transfer rates are expressed as mg. calcium transferred per Kg. per hour. (Rodahl, K., et al: Bone As a Tissue, New York, Blakiston Div., McGraw-Hill)

bone. However, even a prolonged period of calcium deficiency does not lower the blood calcium appreciably;[19] nor does the prolonged negative calcium balance associated with osteoporosis. The two most important factors are the vast reservoir of calcium in the bones,[6] and the function of the parathyroid glands,[8] which, as McLean[21] pointed out, act as "calciostats" to maintain the normal and optimal level of this ion in the blood.

BONE

The skeleton is remarkably well suited to serve this function. On the basis of the cellular material present, it is one of the most vascular tissues in the body, and both the trabeculae and the haversian canals provide a large area of exposure to the circulating blood.[1] It has been estimated on the basis of clearance studies that 4 to 8 per cent of the output of the heart at rest passes through the skeleton,[12] and much higher values are obtained in certain pathologic states, such as Paget's disease. The mineral of bone is also well adapted to provide such a reservoir, for the crystals are of such small size that the relative surface is enormous. From adsorption studies, Neuman[26] has estimated that the total surface area of the crystals of bone salt in the human may be equivalent to 1 to 2 acres. Ion exchange studies with radiocalcium[26] suggest that 10 to 15 per cent of the calcium in ashed bone is exchangeable in vitro and approximately 0.2 to 0.8 per cent in vivo[5] in man. It would seem probable that the latter represents calcium in the hydration shell and on the crystal surfaces of the bone salt accessible to the circulating blood, and that, in fact, this calcium is in equilibrium with the calcium in blood, for the exchange rate is very rapid.[2] If so, this would provide an important labile reservoir of calcium that would tend to "buffer" the blood level.

In addition, there is continual remodeling of the trabeculae and the haversian systems, even in the adult, with attendant bone resorption and new bone formation.[1] Bauer[5] has estimated that the daily accretion of calcium by this means may be as high as 5 Gm. per day. By increasing or inhibiting one or other of these processes, it is possible to change the net movement of calcium into or out of bone and so compensate for changes in the over-all calcium balance.

PARATHYROIDS

The other critical factor in the regulation of the blood calcium is the function of the parathyroid glands. Following parathyroidectomy, the blood calcium level drops, and regulation is grossly impaired,[22] although calcium still can be mobilized from the skeleton.[31] It is now generally agreed that the effect of parathyroid hormone on blood calcium is due to a direct action on bone, as has been demonstrated by Chang[7] by use of gland transplants. Patt and Luckhardt have shown[27,28] that hypocalcemia stimulates parathyroid secretion, and on this basis McLean[21] has suggested that the output of parathyroid hormone is regulated by a feedback mechanism similar to those which control the output of the pituitary hormones. Neuman *et al.*[25] have shown that administration of parathyroid hormone increases the output of citrate by bone and suggests that the calcium-chelating action of the citrate may be responsible for the resulting calcium mobilization from the stable bone mineral.

REGULATION OF BLOOD PLASMA CALCIUM—EXPERIMENTAL

The rapid restoration of a normal plasma calcium has been demonstrated in a number of experiments in which hypocalcemia was induced artificially. Hastings and Huggins,[13] in their classic experiment, carried out replacement transfusions in dogs, using blood from which most of the calcium had been removed by shaking it with lead phosphate. Even when half the blood volume was replaced with this calcium-depleted blood every 10 minutes, it was extremely difficult to lower the blood calcium to a point at which tetany would occur, and, as soon as

the procedure was stopped, the calcium level rose promptly to normal. In the course of one experiment it was estimated that the calcium mobilized from bone was more than four times the amount present in extracellular fluid. The plasma calcium level has also been lowered experimentally by intravenous infusions of oxalate,[27] citrate[29] and disodium ethylenediaminetetraacetate (EDTA).[10,30] In each case the plasma calcium returned quickly to normal once the infusion was stopped.

In this chapter we will report on quantitative studies of the restoration of normal blood calcium levels after intravenous infusions of calcium or EDTA.

Methods

The studies were carried out on normal and thyroparathyroidectomized dogs (maintained on desiccated thyroid) and on adult male subjects admitted to the Clinical Investigation Unit at Shaughnessy Hospital.

The blood calcium was raised by intravenous infusion of calcium as gluconate at a dose of 10 mg. Ca/Kg. body weight, given over a 1-hour period, or it was lowered by infusion of an equivalent amount of EDTA, also over a 1-hour period.

Plasma calcium was determined on samples collected at intervals of 15 to 60 minutes. The method used was the photometric titration with EDTA, as described by Lehmann,[20] using ammonium purpurate as indicator, and a Klett-Summerson photoelectric colorimeter modified by greatly increasing the sensitivity of the galvanometer. Urine calcium was determined by the same method after first precipitating protein and phosphate by the method of Horner.[14] A typical curve for a normal dog is shown in Figure 2. Similar curves were obtained following calcium infusions in normal male subjects.

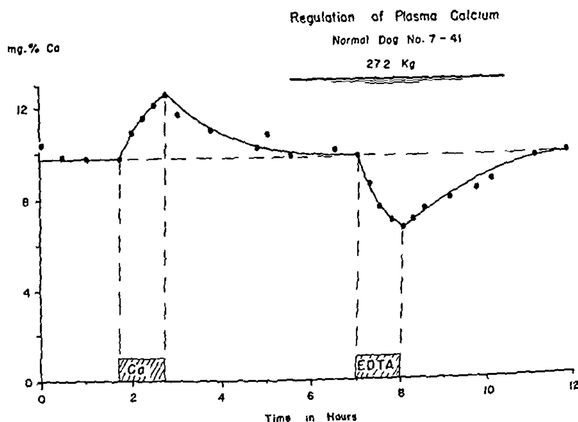


FIG. 2. Changes in level of plasma calcium in a normal dog resulting from infusions of calcium and EDTA (Rodahl, K., *et al.*: Bone As a Tissue, New York, Blakiston Div., McGraw-Hill)

KIDNEY-TM-CA

In all the normal human subjects studied, the renal threshold for calcium was found to be quite close to the fasting control level, and marked calciuria was associated with the hypercalcemia. Above the renal threshold, the increase in excretion rate (UV) was directly proportional to the increase in plasma calcium (p), as shown in Figure 3. These results suggest that there is a definite

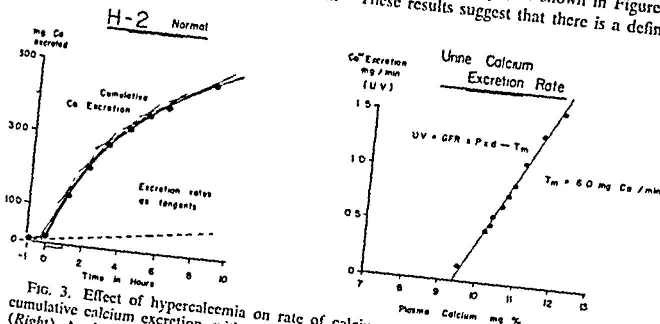


FIG. 3. Effect of hypercalcemia on rate of calcium excretion in urine. (*Left*) Shows cumulative calcium excretion, with tangents drawn to measure excretion rate at any time. (*Right*) A plot of excretion rate (UV) in mg. calcium excreted per minute vs. plasma calcium in mg. %.

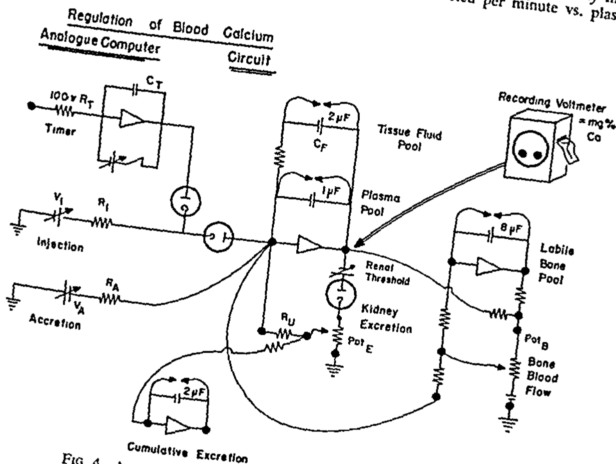


FIG. 4. Analogue computer circuit for simplified calcium system.

tubular maximum (Tm) for calcium reabsorption by the renal tubules in this particular range, which in a series of 10 normal subjects averaged 5.0 ± 0.29 mg.Ca/min. In these individuals, the increased excretion of calcium in urine accounted for 30 to 70 per cent of the injected dose and represented an important factor in bringing down the blood level.

Analogue Computor

By choosing suitable parameters and making certain assumptions, it is possible to set up a system on an electronic analogue computor* comparable with a simplified system of calcium pools and mixing rates in the body. The circuit for such a system is shown in Figure 4. The renal threshold, the Tm-Ca and the slope of the UV vs. p curve are determined experimentally, fixing the values for the components concerned with urinary excretion. It is assumed that the plasma calcium pool is 5 mg./Kg., and the tissue fluid calcium pool is 10 mg./Kg., with half time for mixing of 2 minutes[2]. (This last is not critical.) The only two remaining variables are the labile bone pool and the bone blood flow in the early stages before accretion becomes significant. By varying these, it is possible to obtain a curve that fits the values derived experimentally for plasma calcium. Two typical curves are shown in Figure 5: the lower one represents a normal subject; the upper, a patient with Paget's disease. The computor indicates that the mixing between blood and bone pool is much faster in the case of Paget's disease, as might be expected in view of the tremendous bone blood flow

* Heathkit Electronic Analog Computor, ES-Series.

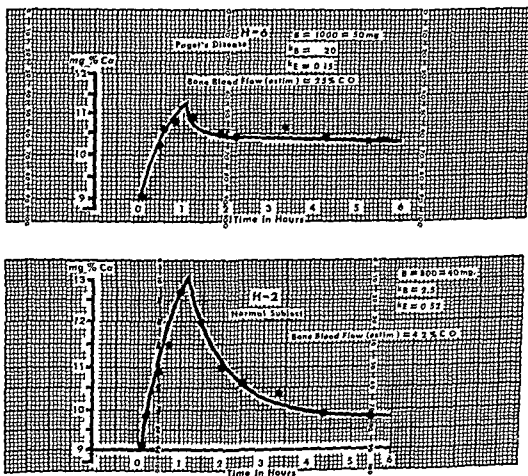


Fig. 5. Plasma calcium determinations matched to analogue computer curves for case of Paget's disease (*above*) and normal subject (*below*).

associated with this condition. While this application of computor analysis is still in an experimental stage and not too definitive, it offers great promise as a means of testing hypotheses relating to calcium regulation, particularly because of the relative simplicity of the calcium system (simplicity that may be more apparent than real).

Parathyroids

As has been observed by McLean and others,[22,23] the fasting plasma calcium level is remarkably constant. The average value of determinations on over 400 blood samples obtained from 100 normal dogs was 9.89 ± 0.54 mg. per cent (2.47 mM./L.), with a standard error of less than 0.3 per cent. However, after total thyroparathyroidectomy, this level fell within 24 to 48 hours to values in the range of 4 to 8 mg. per cent, with an average value on over 150 samples from 42 dogs of 5.84 ± 1.15 mg. per cent (1.46 mM./L.). The standard error in this series was 3.2 per cent, indicative of the very poor regulation of the blood calcium in these animals.

Continuous intravenous infusion of parathyroid extract* at a dose rate of 1 unit/Kg./hr. for 10 to 14 hours was sufficient to restore the plasma calcium level to normal, 0.1 u./Kg./hr. then being sufficient to maintain the normal level (Fig. 6). The latter also appeared to be sufficient to maintain the normal plasma calcium level in a dog following parathyroidectomy, as shown in Figure 7. However, this animal was not able to mobilize additional calcium to overcome the hypocalcemia induced by infusion of EDTA unless additional parathyroid extract was given.

* Parathyroid extract was supplied through the courtesy of E. Lilly and Co.

Continuous I.V. Infusion of Parathyroid Extract

Restoration and Maintenance of
Plasma Calcium Level
Dog No. 7 – 58
(parathyroidectomized 14 days)

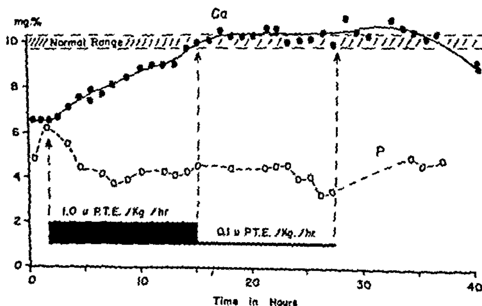


Fig. 6. Effect of intravenous infusion of parathyroid extract on restoration and maintenance of normal plasma calcium levels in a parathyroidectomized dog. (Rodahl, K., et al. Bone As a Tissue, New York, Blakiston Div., McGraw-Hill)

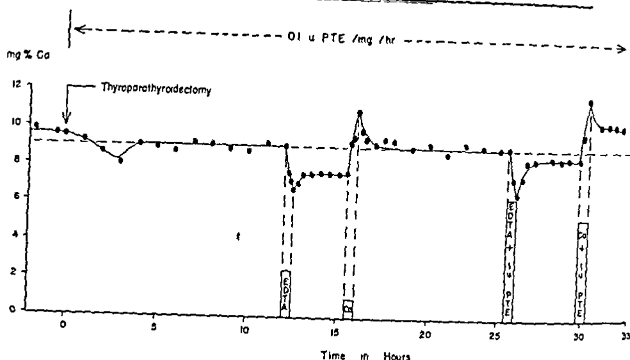


FIG. 7. Maintenance of plasma calcium level following parathyroidectomy by continuous intravenous infusion of parathyroid extract (0.1 u./Kg./hr.). Infusions of Ca and EDTA equivalent to 5 mg. Ca/Kg. over a 30-minute period. (Rodahl, K., *et al.*: Bone As a Tissue, New York, Blakiston Div., McGraw-Hill)

It should be noted that there was a latent period of 20 to 45 minutes following initiation of the parathyroid infusion before a significant increase in plasma calcium was observed, while the effect persisted for some hours after the infusion was stopped This is consistent with the suggestion of Neuman[25] that the parathyroid acts on bone indirectly by stimulating bone cells to produce more citrate.

DISCUSSION

In normal dogs and men there appears to be a very effective homeostatic mechanism that controls the normal plasma calcium level with considerable precision and restores it within a few hours after it has been raised or lowered artificially by intravenous infusions of Ca or EDTA. This involves both the bones and the parathyroids. There is considerable evidence of a labile calcium reservoir in bone in equilibrium with plasma calcium that may correspond to the exchangeable calcium determined with radioactive isotopes. This would act as a "calcium buffer," increasing the effective capacity of the extracellular calcium pool and reducing fluctuations in calcium level. The rate-limiting factor for this process is the bone blood flow. Ultimate restoration of levels will depend on net uptake or release of calcium from stable bone mineral, and it is probable, as McLean[21] has suggested, that this is mediated by the parathyroids through a feedback mechanism. The calciuria associated with hypercalcemia was also an important factor in the normal human subjects, since it accounted for 30 to 70 per cent of the injected dose of calcium.

The situation is somewhat analogous to the regulation of blood sugar, where the liver glycogen provides a reversible storage pool; gluconeogenesis and oxidation represent additions or subtractions from the blood

glucose; and the output of insulin—the principal hormone concerned with homeostasis of blood sugar—is regulated by a feedback mechanism.

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Le Regulation del *Calcium Sanguinee*

Summario in Interlingua

Le mantenentia del concentration optimal del iones de calcium in le liquidos extracellular es un factor de importantia vital in le functionamento normal del tissus. Ille concentration es un del plus cautemente regulate aspectos del plasma sanguinee. Le homeostase del calcium plasmatic depende del vaste reservoir de calcium in le skeleto e del action regulatori del glandulas parathyroide que age como "calciostatos" per medio de un mechanismo de "feedback."

Studios quantitative esseva effectuate in canes e in subjectos human. In illos, calcium esseva infundite intravenosamente (al rhythmo de 10 mg per kg de peso corporee per hora) durante un periodo de un hora o un quantitate equivalente de calcium esseva *eliminate per le infusion de acido ethylenediaminetetraacetic*. Le restauration del normal nivello plasmatic de calcium se effectuava usualmente intra 6 a 10 horas. Le resultatos esseva analysate per medio de un computator analoge electronic, simulante le major reservoirs de calcium e le celeritates de mixtion. Es presentate observationes arguente in favor de un labile reservoir de calcium in osso (possibilemente le excambiabile calcium) le qual se trova in equilibrio con le calcium del plasma. Isto servirea de "tampon" pro le calcium—augmentante le efficace reservoir de calcium extracellular e relaxante o immagasinante lo secundo le requirimentos al momento. Le fluxo de sanguine in osso es un factor importante in le determination del disponibilitate de iste reservoir le qual in humanos pare amontar a inter 2 e 5 g.

Post parathyroidectomia, le calcium del plasma se reduceva ab 9,89 ± 0,54 mg pro cento (i.e. 2,47 mM/L) a 5,84 ± 1,15 mg pro cento (i.e. 1,46 mM/L), e le mantenentia del regulation esseva grossiermente disturbate. Le continue infusion intravenose *de extracto parathyroide* al rhythmo de 1,0 unitates per kg de peso corporee per hora restaurava le nivello normal intra 10 a 14 horas. Un infusion de 0,1 unitates per kg per hora sufficeva pro mantener le nivello normal, sed illo non sufficeva a mobilisar calcium additional como reimplaciamento del calcium removite per le infusion de acido ethylenediaminetetraacetic.

29

Tubular Reabsorption of Phosphorus in Avitaminosis D*

I. SNAPPER, M.D.,† AND A. KAHN, M.D.‡

Whereas formerly the changes of the calcium metabolism in avitaminosis D were emphasized,[11] in later years the hyperphosphaturia and especially the hypophosphatemia have attracted the interest of many investigators.[14] As a matter of fact, the hypophosphatemia is probably the most important factor in the causation of the bone lesion. No marked improvement in the deposition of calcium phosphate can be expected unless the phosphorus concentration of the tissue fluid that surrounds the bone trabeculae rises to normal levels; i.e., unless the hypophosphatemia returns to normal.

* Supported by a grant from the National Heart Institute.

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The hypophosphatemia of avitaminosis D requires careful discussion, because vitamin D does not influence the absorption of phosphorus from the intestine. Therefore, the intestinal phosphorus absorption in avitaminosis D is not impaired and the urinary phosphorus is normal, sometimes even increased. If, however, a patient with avitaminosis D receives large amounts of calcium without additional vitamin D, then unsoluble calcium phosphate forms in the intestine and prevents the absorption of phosphorus. Thus, after ingestion of calcium salts, the phosphaturia of patients with avitaminosis D decreases considerably. (Table 1)[4]* The same holds true of a patient with avitaminosis D treated with iron salts.[5] Hence the

* All experimental results quoted in this chapter have been culled from the Peiping Union Medical College publications mentioned in References[1,4,5-7].

TABLE 1

PERIOD	CA INTAKE mg	P INTAKE mg.	SERUM P mg. %	URINE P mg.	STOOL P mg.
1st	70	818	2 67	319	358
2nd	65	726	2 01	510	187
3rd	1091	748	2 13	324	396
4th	1137	866	1.72	210	541
5th	1090	812	1.59	118	709

In osteomalacia, addition of calcium lactate without vitamin D decreases intestinal P absorption. Stool P increases, urine P decreases
Every period lasts 4 days

(Liu, S. H , Chin, K. Y., Chu, H I., and Pai, H. C : Chinese M. J 58:141)

conclusion that ingestion of calcium without simultaneous administration of vitamin D causes deterioration of the mineral metabolism in avitaminosis D.

The combination of the hypophosphatemia and normal phosphaturia in avitaminosis D has led to many discussions; in later years the role that the kidney plays in the causation of these changes of phosphatemia and phosphaturia has elicited general interest. For the present, it is still accepted that all the phosphate of the plasma is ultrafilterable; i.e., all the phosphates present in the circulating blood appear in the glomerular filtrate. In the proximal convoluted tubule, the greater part—from 80 to 95 per cent—of the phosphate is reabsorbed from the tubular lumen into the blood. It may be added that the normal proximal tubule also reabsorbs 100 per cent of the glucose, the greater part of the amino acids and 99 per cent of the water of the glomerular filtrate.

There are several diseases in which the tubular reabsorption of one or more of these substances are disturbed. The hypophosphatemia combined with hyperphosphaturia present in avitaminosis D is commonly ascribed to impaired tubular reabsorption of phosphate (TRP); i.e., to an abnormally increased clearance of phosphate. Unfortunately, the methods devised to measure the role of the kidney in phosphate metabolism are of recent origin and only became available when the areas in the Orient in which avitaminosis D still is rampant were closed to Occidental scientists. Therefore, no direct calculations are available for the TRP and the phosphate clearance in endemic osteomalacia.

Conclusions based on studies of the metabolism of the avitaminotic rat can hardly be transferred to human rickets and osteomalacia. In contrast with the findings in humans, it is highly probable that, in the rat's lower ileum and proximal colon, absorption of calcium takes place. In addition, the rat develops rickets both on a low-calcium, high-phosphorus diet and on a high-calcium, low-phosphorus diet. This form of rickets can be cured by the feeding of calcium or phosphorus without administration of vitamin D, which is impossible in human avitaminosis D. Finally, the skeleton of the rat is completely different from the human skeleton. In the rat, after occlusion of the epiphysial disks, the epiphysis always continues to contain considerable amounts of cartilage, even if the rat outlives its normal life span. These data emphasize the foolhardiness of using the results of metabolic experiments on the rachitic rat to elucidate the abnormalities of the inorganic metabolism of humans with avitaminosis.

The data of present-day literature that indicate that the tubular reabsorption is impaired in avitaminosis D are rather scanty. This is understandable, seeing that it is seldom that one encounters avitaminosis D nowadays. In addition, some of these data apparently were assembled in order to prove the preconceived concept that an impaired TRP must be present in avitaminosis D. In reading these articles one is reminded occasionally of Goethe's definition of science as "the *artistic** exhibition of facts." Goethe's statement should not be taken lightly, because during his lifetime this poetic author was regarded generally as an excellent scientist. For a century after his death he continued to be appreciated highly as a poet but was decried as a poor amateur in science. Today, he is again considered to be a scientist in his own right, since he is credited with the creation of the foundations of the psychology of vision.[10]

The tendency to subjective evaluation of scientific experiments done to substantiate a preconceived idea makes it advisable to look for objective evidence. For this purpose the authors have restudied the metabolic experiments that the colleagues of one of them (I.S.) of the Department of Medicine of Peiping Union Medical College (originally

* The italics are the authors'.

organized by Dr. Franklin C. McLean) performed 15 to 20 years ago. Their figures, which were collected without any prejudice so far as tubular reabsorption of phosphate was concerned, give valuable evidence; they indicate that in endemic osteomalacia impairment of the TRP actually exists.

The tubular reabsorption of phosphate can be calculated by direct determination of the phosphate clearance and can be approximated by the formula of Stanbury:[11]

$$\text{TRP (in \%)} = \left(1 - \frac{\text{Serum Creatinine} \times \text{Urine P}}{\text{Urine Creatinine} \times \text{Serum P}}\right) \times 100$$

All concentrations are in mg. %.

More important still, Stanbury's analyses have shown clearly that in all patients in whom the serum P is 2.0 mg. % or lower and the 24-hours urinary P exceeds 400 mg., the TRP must necessarily be decreased markedly. Using the latter rule, the figures published 20 and 25 years ago can be scanned in order to find patients with osteomalacia whose serum P varied around 2 mg. %, whereas their daily urinary output amounted to 400 mg. It is hardly necessary to add that in the osteomalacic patients with a serum phosphorus exceeding 2 mg. %, the tubular reabsorption of phosphorus may also have been decreased.

The diets of the population of North China, also of the osteomalacic patients investigated in the metabolic ward of Peiping Union Medical College, were low in total phosphorus.[9] In addition, a good deal of this phosphorus was present in the form of phytic acid of cereals, which cannot be absorbed from the intestine. In the normal person, a low phosphorus intake increases the TRP and decreases the phosphate clearance, in order to save as much phosphorus as possible. Therefore, any decrease in the

TABLE 2

Period	Serum P mg. %	24 Urine mg.	
Patient 1*			
1st & 2nd	1.45	556	
3rd-5th, incl	1.40	406	
6th-9th, incl.	2.70	231	12,000 I.U. vitamin D daily
10th-14th, incl.	2.60	114	12,000 I.U. vitamin D daily
15th & 16th	2 70	42	12,000 I.U. vitamin D daily
Patient 2†			
1st	2.08	375	
2nd	2.01	405	
3rd	2.10	375	
4th	2.50	310	500 I.U. vitamin D daily
5th	3.50	210	500 I.U. vitamin D daily
6th	2 80	110	500 I.U. vitamin D daily
7th	3.60	106	500 I.U. vitamin D daily
8th	3 10	102	
9th-13th, incl.	2 50	200	
13th-23rd, incl	2.40	350	

In osteomalacia, under influence of vitamin D, serum P increases, urine P decreases

* Liu, S. H, Chu, H I., Hsu, H C, Chao, H C, and Cheu, S. H.: J Clin. Invest. 20:267
† Chu, H I., Liu, S H., Yu, T. F, Han, C H, Cheng, T Y., and Chao, H. C.: J. Clin. Invest. 19:352.

TRP of osteomalacic patients kept on a low-phosphorus Chinese diet will be especially significant.

Table 2 reports the data collected in two cases of osteomalacia both before and during treatment with vitamin D. The second patient also was followed after the vitamin D administration had been stopped. In the first patient, in the course of 5 periods of 4 days, the serum phosphorus varied around 1.45 mg. %, whereas the daily phosphorus output was between 406 and 556 mg.[5] There can be no doubt that in this patient, during the 24-day period, the tubular reabsorption of phosphorus must have been impaired markedly and the phosphate clearance increased markedly. The same holds true of the second patient with osteomalacia from Table 2, whose 24-hour urinary output of phosphorus amounted to 405 mg. during a 4-day period, whereas the serum phosphorus had gone down to 2.01 mg. %.[1]

A third example is the patient whose data were reported in Table 1 The table reveals that at one time this patient secreted 510 mg. of P per hour in the urine although her serum P was only 2.01 mg. %.

In the Chinese patients with osteomalacia who were on a constant diet in the metabolic ward of Peiping Union Medical College, the intake of food was supervised very carefully. This guaranteed the stability of the intake and excluded the interference of extrinsic factors, such as changes in the intake of calcium, phosphorus and vitamin D, which could influence the phosphate secretion. It follows from Stanbury's formula that under standard conditions a rise in the serum phosphorus must lead to a rise in the phosphate clearance; i.e, an increase in the urinary phosphate.[13]

In contrast, in nearly all patients with osteomalacia, the administration of vitamin D causes a moderate rise in serum phosphate but a marked reduction in urinary phosphate. In view of the standardized food intake of these patients, this must have been caused by an increase of the TRP; i.e., a decrease in the phosphate clearance. In the first case mentioned in Table 2, vitamin D administered during 36 days led to a gradual increase of the serum phosphorus from 1.40 to 3.07 per cent.* The daily urinary phosphorus, however, declined during the first 16 days of the vitamin D administration from 406 to 231 mg.; during the next 20 days, to 114 mg. In the following 8-day period, under influence of the after-effect of vitamin D, the daily urinary phosphorus output continued to decrease to 42 mg.

In the second patient, a minimal daily dose of 500 I.U. of vitamin D was given for 12 days. The serum phosphorus rose gradually from 2.1 to 3.6 mg. %. In the first 4 days of the vitamin D ingestion, the daily urinary phosphorus output was 310 mg.; in the second 4 days, it was 210 mg.; and in the third 4-day period, 106 mg. During the 4 days after the administration of vitamin D had ceased, the phosphorus output still remained at 102 mg.

These are only two examples of the many experiments published by Peiping Union Medical College[1,4,5,6,7] in which vitamin D caused moderate or marked increase of serum phosphorus combined with marked decrease of phosphaturia. It follows from these observations that in patients with osteomalacia (1) the phosphorus reabsorption in the tubules is impaired and (2) administration of current doses of vitamin D increases the tubular reabsorption of phosphorus.

In later years the moderate hyperplasia of the parathyroids that is found in many patients with avitaminosis D has often been regarded as the cause of these biochemical changes. It was surmised that the hyperplastic parathyroids would excrete an excess of parathyroid hormone. Since this endocrine product actually decreases the tubular reabsorption of the phosphates present in the glomerular filtrate, it would be possible for a so-called secondary hyperparathyroid-

* This value, not recorded in Table 2, is mentioned in the original publication

ism to lead to impairment of the tubular resorption of phosphate. However, the evidence available indicates that in avitaminosis D this is not the case.

Unfortunately, it is impossible to glean data from the older literature to enable us to decide whether or not the parathyroids are responsible for the change of the phosphorus metabolism. Therefore, we must rely on data in present-day literature.[11]

1. In normal persons an intravenous injection of calcium gluconate causes a moderate increase of the calcemia. This allegedly causes an inhibition of the function of the parathyroid glands. Due to the ensuing reduced production of parathyroid hormone, the reabsorption of phosphorus in the renal tubules is less inhibited. Hereby, the serum phosphorus rises and the urinary phosphorus output decreases. In patients with hyperparathyroidism, an intravenous calcium injection is not able to make a dent in the markedly increased secretion of the parathyroids. Thus, in this condition, hypophosphatemia and hyperphosphaturia continue unabated, notwithstanding the injected calcium gluconate. In "primary vitamin D resistant rickets," there exist both a hyperplasia of the parathyroids and a decreased tubular reabsorption of phosphorus comparable with the impaired TRP found in avitaminosis D. In vitamin D resistant rickets, as in the normal person, a calcium infusion changes the phosphaturia into hypophosphaturia, whereas, at the same time, the low-serum phosphorus rises to normal levels.[3] These changes resulting from an increase of the tubular reabsorption of phosphorus persist for 12 to 20 hours; i.e., during the time that the calcium infusion lasts. These results militate strongly against the hypothesis that in primary vitamin D resistant rickets, also probably in avitaminosis D, an appreciable hyperfunction of the parathyroids exists.

2. The same conclusion follows from the observation that after administration of a current dose of 500 to 12,000 units of vitamin D, the tubular reabsorption of phosphorus of the osteomalacic woman increases significantly. The TRP of patients with hyperparathyroidism is not influenced by such relatively small doses of vitamin D.

The actual cause of the decreased TRP in avitaminosis D is not known, even though the work of both Crawford[2] and Saville[8] seems to indicate that a sufficient supply of vitamin D is necessary to guarantee a normal reabsorption of phosphorus by the convoluted tubules. This would explain the reduced TRP leading to hypophosphatemia and hyperphosphaturia commonly observed in avitaminosis D and the return of normal values after administration of vitamin D. Nevertheless, the conclusion that vitamin D has a direct influence on the tubular reabsorption is allegedly not proven, as Stalder[12] could not find a constant improvement of the TRP a few hours after the administration of vitamin D to rachitic children. The increase of the TRP appeared only after a day or longer.

It seems that avitaminosis D leads to complicated alterations of the metabolism of proteins and its building stones and, thereby, to changes in the production of enzymes. The influence of avitaminosis D on protein metabolism is clearly illustrated by the finding that in many cases of infantile rickets, not only hyperphosphaturia, but also aminoaciduria, exists. Thus, avitaminosis D could well change the function of the enzymes that are responsible for the transport mechanism in the renal tubules. When, by the administration of vitamin D, the metabolic changes due to avitaminosis D are repaired, a renewed synthesis of the tubular enzymes would result. This would lead, not immediately, but after a short latent period, to improvement of the tubular reabsorption of phosphorus and other substances.

In this respect it may be of some importance that the impairment of the tubular reabsorption of phosphorus is especially marked in patients with avitaminosis D due to steattorrhea. In such patients where in

addition to fat and fat-soluble substances, considerable amounts of nitrogenous products are lost in the stool, the synthesis of enzymes must be severely damaged.

Another point that constitutes a warning against oversimplification of the problems involved is the increase of resorption of bone *that takes place after administration of* excessive doses of vitamin D: generalized deossification is a characteristic sign of hypervitaminosis D.

SUMMARY

In avitaminosis D the calcium absorption from the intestine is impaired, whereas the phosphorus absorption remains normal. Ingestion of calcium without concomitant administration of vitamin D has an unfavorable influence on the phosphorus metabolism in avitaminosis D.

In avitaminosis D the serum phosphate is always low; the urine phosphorus, always normal, even high. This contrast between serum and urinary phosphorus is due to an impaired reabsorption of phosphorus in the proximal tubules of the kidneys, because (a) patients with osteomalacia may eliminate 400 mg. of phosphorus or even more in the 24-hour urine, although the serum phosphorus had gone down to 2 mg. % or even lower, and (b) in osteomalacic patients, whose intake of calcium, phosphorus and nitrogen was carefully controlled, the increase of phosphorus in the serum under influence of vitamin D was followed by a marked decrease of the urinary phosphorus.

Often, in avitaminosis D, a hyperplasia of the parathyroids occurs. In the opinion of many clinicians, this may well give rise to a secondary hyperfunction of the parathyroids. Experiments are quoted that make it improbable that hyperparathyroidism could be the cause of the impaired tubular reabsorption of phosphorus in avitaminosis D.

It seems possible that avitaminosis D *could reduce the synthesis of protein and thereby impair the synthesis of the enzymes* that are responsible for the reabsorption of *phosphorus by the renal tubules.*

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Reabsorption Tubular de Phosphoro in Avitaminosis D

Summario in Interlingua

Le problemas de avitaminosis D concerne le metabolismo de calcium e etiam de phosphoro In plus recente annos, le importantia del alterationes in le metabolismo de phosphato ha attrahite plus attention

In avitaminosis D, le reabsorption de cal-

cium ab le intestino es imperfecte, durante que le absorption de phosphoro remane intacte. Le ingestion de calcium sin administration concomitante de vitamina D ha, in casos de avitaminosis D, un influentia disfavorabile super le metabolismo de phosphoro.

In avitaminosis D, le ingestione de altere cationes que forma insolubile sales phosphatic—specialmente sales de ferro e de aluminium—causa le mesme imperfection del absorption intestinal de phosphato como le ingestion de calcium.

In avitaminosis D, le concentration de phosphato in le sero es semper basse, illo in le urina es semper normal o mesmo elevate. Iste contrasto inter le phosphato del sero e le phosphato del urina es le effecto de un imperfection del reabsorption de phosphoro in le tubulos proximal del renes. Iste assertion se basa super le sequente constatationes:

1. Patientes con osteomalacia pote eliminar 400 mg. de phosphoro o mesmo plus in le urina de 24 horas, ben que le phosphoro in lor sero ha descendite a 2 mg. per cento ml. o plus basse ancora.

2. In patientes con osteomalacia, in qui le ingestion de calcium, phosphoro, e nitrogeno esseva cautemente regulate, le augmento del phosphoro in le sero occurrente sub le influentia de vitamina D esseva sequite per un marcate reduction del phosphoro in le urina. Isto pote indicar nihil altere que le facto que sub le influentia de vitamina D le imperfecte reabsorption tubular de phosphoro retorna al stato de normalitate.

In avitaminosis D, hyperplasia del glandulas parathyroide occurre frequentemente. Multe clinicos opina que isto pote ben esser responsabile pro le disveloppamento de un hyperfunction secundari del parathyroides. Es citate experimentos que rende improbabile le notion que hyperparathyroidismo pote esser le causa del imperfecte reabsorption tubular de phosphoro in avitaminosis D. Etiam le sequente observationes debe esser prendite in consideration:

1. Le injection intravenose de calcium in patientes con rachitis resistente a vitamina D causa un augmento del reabsorption tubular de phosphoro. Iste phenomeno non occurre quando le mesme injectiones de calcium es administrate a patientes qui suffre de hyperparathyroidismo.

2. Le doses currente de vitamina D (i.e., 500 a 1,000 unitates) suffice a renormalisar le imperfecte reabsorption tubular de phosphoro in avitaminosis D. Isto es nettemente non le caso in patientes con hyperparathyroidismo.

Il pare que un adequate provision de vitamina D es necessari pro garantir le reabsorption normal de phosphoro in le proximal tubulos convolute. In iste connexion il es necessari signalar que avitaminosis D resulta etiam in alterationes del metabolismo de proteina, un facto evidentiate per le aminoaciduria que occurre in avitaminosis D. Il pare possibile que avitaminosis D reduce le synthese de proteina e distruba assi le synthese del enzymas que es responsabile pro le reabsorption de phosphoro per le tubulos renal.

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Metabolic Studies in Cushing's Syndrome*

The Effect of Steroid Withdrawal, Androgen and Vitamin D on Calcium, Phosphorus and Nitrogen Balance

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The identification and the synthesis of adrenocortical hormones and certain of their analogues have led to development of highly effective replacement therapy in adrenal insufficiency. Complete adrenalectomy now can be performed on man with reasonable assurance of recovery and maintenance of health.[18] In fact, current practice appears to favor total removal of the adrenals when Cushing's syndrome occurs as a consequence of bilateral cortical hyperplasia.[39] Since osteoporosis is a frequent manifestation of more severe forms of this disorder,[45] it was hoped that a study of calcium, phosphorus and nitrogen balances during recovery from hyperadrenocorticism might help to clarify the postulated relationship between the adrenal cortex and calcium homeostasis.[21,57,51,40,45,42,36,33] Although a brief investigation along these lines was reported previously by Kepler *et al.*,[32] the authors are unaware of any prolonged observations subsequent to total ablation of the adrenals.

The present investigation deals with two individuals: (1) D.M., a man of 24 who was adrenalectomized after it had been established by careful clinical studies that he had Cushing's syndrome; (2) H.S., a man of 45 with pemphigus vulgaris in whom attempts at withdrawal of corticosteroid therapy induced symptoms and signs of steroid withdrawal. This man, because of continuous medication with steroids for several years, had developed many of the signs of hyperadrenocorticism. As a result of the metabolic studies, it can be shown (a) that the symptoms of steroid withdrawal in each case were associated with a marked increase in urinary calcium and a negative calcium balance and (b) that the abnormalities of calcium exchange appeared to be of a temporary nature and occurred in the presence of strongly positive nitrogen and phosphorus balances.

A possible antagonism between cortical steroid therapy and vitamin D was looked for in the second subject. However, none was observed. In fact, the data obtained indicated intoxication with vitamin D manifested by hypercalciuria at dosage levels of 50,000 I.U. per day; hence large doses of D appeared to be contraindicated.

CASE D.M.

A white 24-year-old laborer, employee of the Santa Fe Railroad. This man was admitted to Wadsworth General Medical and Surgical Hospital Veterans Administration Center, Los Angeles, on April 17, 1957. Approximately 4 weeks prior to his admission he

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had applied for another job. On pre-employment physical examination, although he claimed to have felt perfectly well, the physician noted that his face was rounded and plethoric, that he had a "buffalo hump," that there were striae over the hips and the shoulders, and that the blood pressure was elevated. The plethoric appearance had been apparent for approximately 1½ years. He denied headache, malaise, bone pain, loss of body hair or loss of libido. His own past history and his family history were noncontributory.

Physical Examination

Height, 173 cm.; weight, 77 Kg.; temperature, 37° C.; pulse, 90; blood pressure, 210/105 mm. Hg. The patient's general condition appeared to be excellent. Positive findings were as follows: hypertension as noted; suggestive increase in the suprascapular fat pad; rounded plethoric face; moderate reddish striae over both hips and shoulders; normal amount and distribution of body hair; eyes—hyperopia O.D., compound hyperopia O.S., amblyopia exanopsia O.S., ocular fundi, very slight attenuation of retinal arterioles. Otherwise, no significant findings.

Laboratory Data

Hemogram. Hemoglobin, 18 Gm./100 ml. whole blood; hematocrit, 54 vols. % packed cells; white blood cell count, total leukocytes 11,100; differential count—neutrophils 55 per cent, lymphocytes 16 per cent, monocytes 5 per cent, eosinophils 1 per cent; platelets adequate in numbers.

Routine Urinalysis. pH, 6; specific gravity, 1.027; protein, none; sugar, none; microscopic, 5RBC's and 3WBC's per high power field centrifuged specimen.

Serologic Test. For lues (VDRL), negative.

Blood Chemistry. Serum electrolytes mEq./L.: sodium, 138; potassium, 5.3; chlorides, 95; CO_2-combining power, 18.4; calcium, 9.9 mg. %; inorganic phosphorus, 3.3 mg. %;

Plasma cholesterol, total 247 mg. %.

Serum protein bound iodine, 5.7 mcg. %.

Fasting blood sugar, 48 mg. %. Creatinine, 1.0 mg. %;

Glucose tolerance test (3 hrs.-100 Gm. glucose orally) blood sugar: fasting, 91 mg. %; 30 minutes, 134 mg. %; 1 hour, 147 mg. %; 2 hours, 117 mg. %; 3 hours, 100 mg. %. No glucose was excreted in the urine during test.

Test of Response of Adrenal Cortex to Stimulation and Suppression. Urinary Steroids 24-Hour Excretion

Date	17-Keto Steroids	17-Hydroxy Steroids	Medication
4-18-57	33.8 mg.	13 4 mg.	
4-21-57	22.0 mg.	9.8 mg	
4-23-57	32.9 mg.	17.1 mg.	
4-26-57			3-day ACTH test 20 units ACTH in 5% glucose 1,000 ml. given I.V. over 8-hr. period: 4-26; 4-27; 4-28.
4-27-57	59.2 mg.	58 3 mg.	
4-28-57	66.0 mg.	24.6 mg.	
5- 5-57	44.2 mg.	22.0 mg.	No medication
5- 6-57			Adrenal suppression test: 5 mg 9 alpha fluoro delta[1] hydrocortisone* 5-6-57 through 5-10-57.
5-10-57	37.9 mg	21.8 mg	
5-11-57	37.7 mg.	20 2 mg	
7-19-57	54.5 mg.	25.0 mg.	

* Hereinafter referred to as 9 alpha F

The eosinophil count revealed 33 cells per cm. on the 3rd day of suppressive therapy with 9-α-fluoro-Δ' hydrocortisone (9 alpha F). No eosinophils were seen 3 days after cortical suppression was discontinued. Radioactive iodine uptake, 14 per cent in 6 hours, 22 per cent in 24 hours. Urinary calcium collection made during intravenous ACTH test, 838 mg. in 24 hours; urine collected just prior to adrenocortical suppression test with 9 alpha F, 365 mg. in 24 hours.

Roentgen Examinations. SKULL roentgenograms taken April 17, 1957. The bony calvarium was intact. No abnormality of vascular pattern was seen. The size of the sella turcica and of the clinoid processes was normal in appearance. The petrous ridges were intact. No abnormal densities or calcifications were noted.

ROENTGENOGRAM OF CHEST. The cardiac silhouette was slightly enlarged. The great vessels appeared to be normal. Mediastinal shadows were moderately prominent. There were a few small well-calcified densities in the lung parenchyma.

PYELOGRAM RETROGRADE TAKEN MAY 8, 1958. The renal calyces, the pelvis and the ureters appeared to be normal.

REPEAT SKULL SERIES TAKEN JULY 9, 1959. The findings did not differ from those of April 17, 1957.

BONE SURVEY MADE APRIL 25, 1957. There was a question of some minimal demineralization of the lumbar spine, otherwise there was no evidence of bone or joint disease.

Transfer to the Metabolic Research Unit was made on May 21, 1957. The results of the studies conducted during this phase of admission are discussed in the main body of the report.

The patient was transferred to the Surgical Service on September 4, 1957. As preliminary preparation for complete bilateral adrenalectomy, he received cortisone acetate, 100 mg. intramuscularly, at 8:00 P.M. on September 4 and at 6:00 A.M. on September 5. During the operation, 100 mg. of hydrocortisone hemisuccinate, dissolved in 1 liter of 5 per cent dextrose in water, was administered intravenously.

OPERATIVE NOTE (DR. JAMES CLARKE)

The peritoneal cavity was opened through an incision made from the angle of the right 12th rib and was carried forward to the anterior axillary line. After exploration by touch it was ascertained that both the right and the left kidneys were present. No adrenal tumor could be palpated in either the right or the left suprarenal area. The peritoneum then was closed, and the right 12th rib was resected to a point about 4 cm. lateral to the mid-line posteriorly. The right adrenal gland was freed from its surrounding attachments by dissection but was fractured several times in the process. The entire right gland was about normal in size and consistency, and was removed completely. The wound was closed. The patient was turned on his right side, and a similar approach was used to expose the left adrenal. This also was removed completely. Neither pleural cavity was opened during the course of the operation. Bleeding was not excessive, and both wound areas were dry at the completion of the procedure. The patient withstood the operation well, and the systolic blood pressure remained over 130 mm. Hg throughout. On inspection it was felt that the left adrenal gland was moderately enlarged; however, there was no evidence of tumor in either gland.

NOTE BY PATHOLOGIST
(DR. LOUIS LICHTENSTEIN)

The left adrenal gland was fragmented. When freed from fat it measured 7 x 3 cm. and weighed 15 Gm. The right adrenal gland measured 8 x 3 cm and weighed 22 Gm. with some fat attached. Microscopic appraisal: both adrenal glands showed considerable diffuse cortical hyperplasia. No distinct adenoma was present in either gland.

POSTOPERATIVE TREATMENT

During the immediate postoperative period the same dose of hydrocortisone hemisuccinate was repeated. On September 6 the patient received 50 mg. of cortisone acetate intramuscularly every 6 hours for 3 days. On the 4th day, cortisone acetate was reduced to 50 mg., given intramuscularly at intervals of 8 hours. By the 6th day, oral medication again was possible, and the dose of cortisone was reduced further to 25 mg. every 6 hours (100 mg. per day). This schedule of dosage was continued through September 12, when the time interval was rearranged so that 25 mg. was administered at 6:00 A.M., 11:00 A.M., 5:00 P.M. and 9:00 P.M. The patient returned to full balance program on the 6th postoperative day. Further changes in specific medication appear in the figures and the tables. It is to be noted that he made a satisfactory recovery without the inclusion of a salt-active corticoid in his medication, and it was not considered to be necessary to give 9 alpha F until cortisone had been reduced to 50 mg. daily. At this time the patient developed arthralgia, weakness, headache and loss of appetite. Subsequently these were thought to be due to the decrease in the dose of cortisone. The main features of the succeeding 220 days of his postoperative course are recorded in the figures and the tables dealing with the postoperative metabolic study.

POSTOPERATIVE URINARY STEROIDS

1957	Urine Vol ml	17-Keto Steroids mg	17-Hydroxy Steroids mg
9- 6 to 9- 7 . . .	600	17 3	not done
9- 7 to 9- 8 .	625	11	12.5
9- 8 to 9- 9 . . .	600	6.2	9 0
9- 9 to 9-10 . . .	850	17 5	16 8
10-10 to 10-11 . . .	1650	15 8	13 2
10-31 to 11- 1 . . .	1590	8 4	6 2
11- 7 to 11- 8 . . .	1293	10.0	6 5
11-14 to 11-15 . . .	1605	9 9	5 4
12-19 to 12-20 .	1860	9 2	5.8

April 26, 1958. Following completion of the balance studies the patient was discharged to the Outpatient Clinic and readmitted about 1 month later to determine the completeness of adrenalectomy. His general physical condition remained good.

Medication. Cortisone, 25 mg. twice a day; 9 alpha F, 125 mcg. daily.

Blood. HEMOGLOBIN, 15.9 Gm. %; hematocrit, 48 vols. % packed cells; white blood count, 6,800; sedimentation rate, 5 mm./hr. (Wintrobe); serum sodium, 142 mEq./L; serum potassium, 5.2 mEq./L; chloride, 101 mEq. L; CO_2, 23.7 mEq./L; fasting blood sugar, 38 mg. %.

URINALYSIS. Specific gravity, 1.026; pH, 5.0; protein, negative; sugar, negative; microscopic, negative.

Course in Hospital. Cortisone was disdiscontinued, but the patient received his regular dose of 9 alpha F. On May 22, 1958, 24 hours after cortisone was discontinued, he was given an infusion of 1 L. 5 per cent dextrose and water containing 25 units of ACTH over a 4-hour period. The concentration of plasma 17-hydroxycorticoid was determined before and after the infusion. The control level of plasma corticoid was 0 mcg. % and rose to 2.6 mcg. % in the postinfusion period. These values were regarded as being within the limitations of the method and not as indicating any stimulation of adrenocortical tissue by ACTH. Water-loading tests also demonstrated impairment of adrenal function. After the completion of these tests, treatment was reinstituted with hydrocortisone—20 mg. twice daily—and the malaise and nausea induced by omission of glucocorticoid disappeared promptly.

December 16, 1958. Roentgenogram of lumbar spine: bone density was normal and disk spaces were well preserved; numerous metallic clips overlay the renal areas.

CASE H.S.

A white 50-year-old male of Mexican parentage. During his period of military

service in the Philippines in 1945, lesions of the skin, the face and the trunk developed. Some of these were crusted, others bullous in nature. After his discharge from the Army, he continued to work as a Sharpless operator in a vegetable oil plant until he was admitted for the first time to the Wadsworth General Medical and Surgical Hospital of the Los Angeles facility on June 12, 1951, for essentially the same type of skin disease as that noted during military service. After 4 months of careful study and observation on the Dermatology Service, and as a result of biopsies of skin lesions, a diagnosis of pemphigus vulgaris was made.

Physical Examination

Height, 165 cm.; weight, 77 Kg.; blood pressure, 126/90 mm. Hg; Temperature, 37° C.; pulse, 80. His general appearance was that of a rather short, intelligent, well-preserved man. The main point in the examination related to the skin. A scaling, crusted erythematous lesion about 2½ cm. in diameter was present beneath the right eye. There were many rounded, crusted, slightly erythematous lesions over the chest and the back, varying in size from 0.5 to 1.5 cm. in diameter, as well as several similar but smaller lesions on the scalp and a few scattered bullae on the back and the chest. The mucous membranes were not involved. No abnormalities were discovered on examination of the heart, the lungs, the extremities and the nervous system Rectal examination revealed adequate sphincter tone; the prostate was normal in size and consistency; moderately enlarged internal hemorrhoidal veins were present and bled easily.

Laboratory Data

Hemogram. RBC's, 5.1 million per cu. mm.; hemoglobin, 15 Gm./100 ml. whole blood; hematocrit, 47 vols. % packed cells; sedimentation rate, 16 mm./hr. (Wintrobe); WBC's, 6,100 per cu mm.; differential count—neutrophils 49 per cent, lymphocytes 35 per cent, monocytes 6 per cent, eosinophils 8 per cent, basophils 2 per cent.

L. E. Cells. Negative on two occasions.

Sternal bone marrow shows normal distribution of myeloid elements.

Serology. Wassermann and Kahn reactions negative.

Routine Urinalysis. Specific gravity, 1.023; pH, 6.0; protein, negative; sugar, negative; microscopic, rare WBC; few squamous epithelial cells.

Blood Chemistry. SERUM ELECTROLYTES mEq./L. Sodium, 138; potassium, 4.9; chloride, 100; CO_2-combining power, 27.6; calcium, 9.9 mg. %; inorganic phosphorus, 3.8 mg. %; alkaline phosphatase, 4 0 Bodansky units.

PLASMA PROTEINS (Tiselius electrophoresis). Total protein, 6.14 Gm. %; albumin, 3.0 Gm. %; total globulins, 3.14 Gm. %; alpha globulin, 0.89 Gm. %; beta globulin, 1.09 Gm. %; gamma globulin, 0.63 Gm %; serum protein bound iodine, 8.7 mcg. %.

THYROID UPTAKE OF RADIOACTIVE IODINE (2 μc. CARRIER FREE ORALLY). 6 hours, 3 per cent; 24 hours, 5 per cent; 48 hours, 5 per cent (depression of uptake thought to be due to medication with cortisone).

URINARY 17 KETOSTEROID EXCRETION. 7.3 mg./24 hrs. and 6.0 mg./24 hrs.

RENAL FUNCTION, January 11, 1955. Plasma creatinine, 1.39 mg. %; urine creatinine, 2.02 Gm./24 hrs.; creatinine clearance, 100.6 ml./min.

Skin Tests for tuberculosis, blastomycosis, coccidioidomycosis and histoplasmosis were negative.

Course in Hospital

After numerous attempts to determine the dose of corticosteroid that would suppress the skin lesions satisfactorily, this was finally established as 125 mg. of cortisone daily. However, as a result of steroid therapy, the peculiar fat distribution, the rubicundity and the round plethoric facies of Cushing's syndrome developed.

At the end of the 2nd year on cortisone (November, 1954), roentgenograms of the dorsal and the lumbar spine showed evidence of generalized bone demineralization. The vertebral bodies remained in good alignment with intervertebral spaces well maintained.

In May, 1956, cortisone was discontinued, and prednisone in doses of 25 mg. daily was substituted successfully.

The balance study reported here began in February, 1957, and was continued for 8 months. Only one brief illness required an interruption of 2 days. On June 3 the patient complained of pain in the low back. There were lumbar tenderness and fever of 40.5° C. Clumps of white blood cells were found in the urine; the leukocyte count was 10,200, with 94 per cent neutrophils. Urine culture showed a heavy growth of *Proteus mirabilis*. Rapid recovery followed a regimen of bed rest, forced fluids and medication with tetracycline.

Subsequent to completion of the balance, an attempt was made to maintain the patient on prednisone, reduced to 5 mg. daily. The low-dosage schedule proved to be inadequate, and more numerous and frequent crops of bullae began to appear, necessitating an increase in the drug to 15 mg. daily. Six-methyl-prednisolone also proved to be ineffective in doses of 6 mg. daily.

Rather frequent complaints of back pain, confirmation of some demineralization of the spine by recent roentgenograms, plus evidence from metabolic studies that anabolic steroids decreased calcium excretion in the urine moderately, were responsible for initiation of long-term therapy with testosterone enanthate, 90 mg., and estradiol valerate, 4 mg These steroids have been administered on a biweekly schedule by intramuscular injection.

Biopsy of bone from the region of the right greater trochanter in February, 1959, gave the impression that the femoral shaft was somewhat softer and more brittle than normal Cancellous bone obtained from the trochanter was considered to be osteoporotic by the pathologist. Whether or not anabolic steroid therapy has slowed the progress of demineralization is unknown. Equally important may be the fact that corticoid dosage has been kept at a minimum commensurate with relative freedom from the lesions of pemphigus, and that the man has been encouraged to ingest a diet high in protein, calcium and phosphorus, which in his case appears to maintain a state of equilibrium.

PROCEDURES AND METHODS

The patients were admitted to the metabolic ward, where they were kept under careful observation by specially trained personnel. Weighed diets were prepared under the supervision of the dietitian in charge of the special ward kitchen. Both subjects were allowed some initial latitude in the choice of foods, but, once the program began, only minor changes as noted below were permitted. Every 10th day a duplicate diet was prepared for each patient, and this was analyzed for nitrogen, calcium and phosphorus. The results of these analyses were used to determine the intakes of these substances. Urine was kept under refrigeration, and each 24-hour collection was analyzed for nitrogen and phosphorus. Aliquots were pooled in 5-day periods, and calcium was determined on each pool unless otherwise specified. Five-day collections of stools were saved for analyses. No markers were used, since the authors are of the opinion that, in general, little is to be gained by this procedure. The fecal values given in tables and in charts are the result of averaging analytic data obtained in several consecutive metabolic periods. Blood was drawn usually at the beginning of each metabolic period with the patients always in the postabsorptive state. The analytic methods were as follows:

Aliquots of diets, urine and stool were ashed in a muffle furnace at a temperature not exceeding 450° C. Solutions of the ash were used in the determination of calcium

and phosphorus in diet and in stool. Calcium also was determined on solutions of ashed urine. Total nitrogen was determined by macro Kjeldahl on each 24-hour collection of urine and on homogenates of diet and feces. Calcium in the urine, the feces and the diet was determined by the gravimetric method of Washburn and Shear;[62] calcium in serum by the procedure of Kochakian and Fox.[37] The analyses for phosphorus were made by the method of Fiske and Subarrow,[17] with slight modifications to adapt it for the photoelectric colorimeter. Total urinary 17-ketosteroids were determined by the method of Drekter *et al.*;[16] 17-hydroxycorticoids in urine by Reddy's modification of the Reddy-Jenkins-Thorn method.[46]

D.M. received a diet calculated to yield approximately 2,200 calories: protein, 98 Gm.; carbohydrate, 202 Gm.; fat, 113 Gm. After 15 days he appeared to be losing weight slowly. Therefore, the carbohydrate in the food was increased to 288 Gm. daily, raising the intake to 2,530 calories. This small change in calories was sufficient to maintain weight equilibrium. Actual analyses of sample diets during the 19 control periods (96 days) revealed mean daily intakes of nitrogen, 16.62 Gm.; calcium, 1,147 mg.; phosphorus, 1,607 mg. (Table 1). Owing to a change in the source of milk, a small increase in both calcium and phosphorus occurred in Periods 8 through 19 and has been taken into account in computing balances (Figs. 1 & 2 and Table 1).

On completion of the preliminary observations the patient was transferred to the Surgical Service, where a complete adrenalectomy was performed. Six days subsequent to the operation he was able to resume the routine of the metabolic ward. A minor adjustment in the composition of the diet was made at this time (Period 20). Essentially, calcium, phosphorus, protein and calories remained unchanged. Fat was increased from 113 to 138 Gm. and carbohydrate was reduced from 288 to 230 Gm. daily. Analyses of sample diets—periods 20 through 63—give mean daily values for nitrogen of 16.98 Gm.; calcium, 1,215 mg.; phosphorus, 1,699 mg. (Table 1).

TABLE 1. COMPOSITION OF DIETS (MEAN DAILY INTAKES)

Subject	Periods	CHO Gm. Approx.	Fat Gm. Approx.	Protein Gm. Approx.	Calories Gm. Approx.	*Calcium mg.	*Phosphorus mg.	*Nitrogen Gm
D.M.	1- 3	202	113	98	2200	1147 ± 14 (4)	1607 ± 24 (4)	16.62 ± 0.28 (10)
	4- 7	288	113	98	2530	1147 ± 14 (4)	1607 ± 24 (4)	16.62 ± 0.28 (10)
	8-19	288	113	98	2530	1196 ± 10 (6)	1697 ± 47 (6)	16.62 ± 0.28 (10)
					Postoperative			
	20-63	230	138	98	2530	1215 ± 29 (22)	1699 ± 46 (22)	16.98 ± 0.30 (22)
H.S	1-25	212	89	129	2130	1130 ± 20 (13)	1914 ± 33 (13)	21.10 ± 0.45 (13)
	26-45	212	89	129	2130	1000 ± 23 (9)	1831 ± 45 (9)	20.14 ± 0.48 (10)

Estimates of carbohydrate, fat and protein are based upon standard dietary tables; values marked with an asterisk are means obtained by analysis of sample daily menus weighed out by dietitian. Figures in parentheses below each mean indicate number of sample diets analyzed; ± refers to one standard deviation from the mean.

and phosphorus in diet and in stool. Calcium also was determined on solutions of ashed urine. Total nitrogen was determined by macro Kjeldahl on each 24-hour collection of urine and on homogenates of diet and feces. Calcium in the urine, the feces and the diet was determined by the gravimetric method of Washburn and Shear;[62] calcium in serum by the procedure of Kochakian and Fox.[37] The analyses for phosphorus were made by the method of Fiske and Subarrow,[17] with slight modifications to adapt it for the photoelectric colorimeter. Total urinary 17-ketosteroids were determined by the method of Drekter *et al.*,[16] 17-hydroxycorticoids in urine by Reddy's modification of the Reddy-Jenkins-Thorn method.[46]

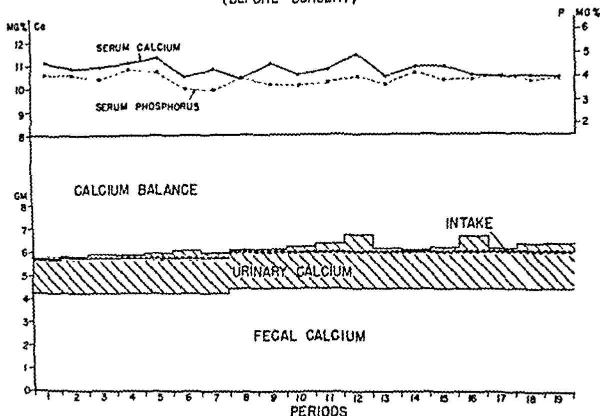
D.M. received a diet calculated to yield approximately 2,200 calories: protein, 98 Gm.; carbohydrate, 202 Gm.; fat, 113 Gm. After 15 days he appeared to be losing weight slowly. Therefore, the carbohydrate in the food was increased to 288 Gm. daily, raising the intake to 2,530 calories. This small change in calories was sufficient to maintain weight equilibrium. Actual analyses of sample diets during the 19 control periods (96 days) revealed mean daily intakes of nitrogen, 16.62 Gm.; calcium, 1,147 mg.; phosphorus, 1,607 mg. (Table 1). Owing to a change in the source of milk, a small increase in both calcium and phosphorus occurred in Periods 8 through 19 and has been taken into account in computing balances (Figs. 1 & 2 and Table 1).

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TABLE 1. COMPOSITION OF DIETS (MEAN DAILY INTAKES)

Subject	Periods	CHO Gm Approx.	Fat Gm. Approx	Protein Gm. Approx.	Calories Gm. Approx.	*Calcium mg.	*Phosphorus mg.	*Nitrogen Gm
D M	1- 3	202	113	98	2200	1147 ± 14 (4)	1607 ± 24 (4)	16 62 ± 0 28 (10)
	4- 7	288	113	98	2530	1147 ± 14 (4)	1607 ± 24 (4)	16.62 ± 0.28 (10)
	8-19	288	113	98	2530	1196 ± 10 (6)	1697 ± 47 (6)	16.62 ± 0 28 (10)
					Postoperative			
	20-63	230	138	98	2530	1215 ± 29 (22)	1699 ± 46 (22)	16.98 ± 0 30 (22)
H.S.	1-25	212	89	129	2130	1130 ± 20 (13)	1914 ± 33 (13)	21 10 ± 0.45 (13)
	26-45	212	89	129	2130	1000 ± 23 (9)	1831 ± 45 (9)	20 14 ± 0 48 (10)

Estimates of carbohydrate, fat and protein are based upon standard dietary tables, values marked with an asterisk are means obtained by analysis of sample daily menus weighed out by dietitian. Figures in parentheses below each mean indicate number of sample diets analyzed; ± refers to one standard deviation from the mean.



FIGS. 1 to 6, Case D.M. FIG. 1. *Preoperative serum calcium, inorganic phosphorus and calcium balance* Calcium balance recorded in periods of 5 days each; urinary calcium per period indicated as hatched area above fecal calcium (clear area); mean values for fecal calcium (Periods 1-7 & 8-19) have been plotted. Mean calcium intake shown by solid line ± one standard deviation (dotted lines). Projection of urinary calcium above intake line denotes negative balance. Note that two mean intakes have been computed (Periods 1-7 & 8-19) *due to change of source of milk in diet.*

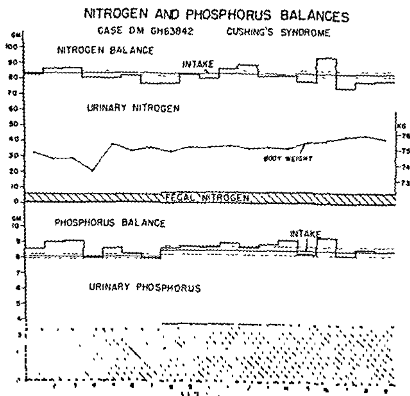
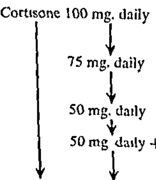


FIG 2. Preoperative nitrogen and phosphorus balances. Graph prepared as in Figure 1 *except that hatched areas denote fecal excretions and clear areas excretion in urine. Body weight—right-hand scale —superimposed on urinary nitrogen.* Where sum of urinary and fecal excretions fall below intake line, balance is positive; extension of sum above intake line denotes negative balance.

TABLE 2. CALCIUM, PHOSPHORUS AND NITROGEN BALANCES, SUBJECT D.M. HEIGHT 173 CM.

Period (5 Days)	Body Wt. Kg.	Procedures and Medication	Calcium, mg. Urine mg.	Calcium, mg. Stool mg.	Calcium, mg. Balance mg.	Phosphorus, mg. Urine mg.	Phosphorus, mg. Stool mg.	Phosphorus, mg. Balance mg.	Nitrogen, Gm. Urine Gm.	Nitrogen, Gm. Stool Gm.	Nitrogen, Gm. Balance Gm.
1	74 68		1,463	4,224	+ 47	5,218	3,310	− 492	77.10	6.11	− 0.10
2	74.44		1,598	4,224	− 88	5,649	3,310	− 923	80.62	6.11	− 3.62
3	74 48		1,691	4,224	− 181	5,725	3,310	− 999	81.09	6.11	− 4.09
4	73 82		1,664	4,224	− 154	4,655	3,310	+ 71	74.88	6.11	+ 2.12
5	72.40		1,738	4,224	− 228	5,267	3,310	− 541	74.55	6.11	+ 2.45
6	74 95		1,860	4,224	− 350	4,914	3,310	− 188	76.20	6.11	+ 0.80
7	75.03		1,748	4,224	− 238	4,631	3,310	+ 95	70.63	6.11	+ 6.37
8	74 97		1,679	4,425	− 122	4,888	3,730	− 136	70.11	6.11	+ 6.89
9	74 88		1,694	4,425	− 137	4,991	3,730	− 239	75.80	6.11	+ 1.20
10	74.96		1,774	4,425	− 217	4,938	3,730	− 186	73.17	6.11	+ 3.83
11	75 12	Glucose tolerance test	1,930	4,425	− 373	5,222	3,730	− 470	79.37	6.11	− 2.37
12	75 14	Calcium I.V. as gluconate	2,314	4,425	lost	4,932	3,730	− 180	81.86	6.11	− 4.86
13	74.94		1,721	4,425	− 164	5,154	3,730	− 402	74.77	6.11	+ 2.23
14	75 14		1,669	4,425	− 112	5,421	3,730	− 669	75.36	6.11	+ 1.64
15	75.16		1,758	4,425	− 201	4,527	3,730	+ 225	71.11	6.11	+ 5.89
16 (6 days)	75 52		2,269	5,310	− 403	5,600	4,476	+ 102	86.58	6.11	+ 5.82
17	75.98		1,690	4,425	− 133	4,465	3,730	+ 287	67.42	6.11	+ 9.58
18	75.78		1,900	4,425	− 343	4,868	3,730	− 116	71.26	6.11	+ 5.74
19	75 68		1,910	4,425	− 353	4,754	3,730	− 2	71.97	6.11	+ 5.03
		Operation—Balance Discontinued for 6 Days									
20	73 35	Cortisone 100 mg. daily	1,698	4,946	− 659	4,427	3,969	+ 99	73.50	6.51	+ 4.89
21	72.97		1,564	4,946	− 435	4,218	3,969	+ 308	67.05	6.51	+11.34
22	72.36		1,477	4,946	− 348	4,461	3,969	− 135	65.57	6.51	+12.82
23	72.57	75 mg. daily	1,240	5,230	− 395	4,461	3,964	+ 70	65.10	6.51	+13.29
24	72.75		1,138	5,165	− 228	4,302	3,723	+ 470	64.71	6.51	+13.68
25	73.15		1,082	5,230	− 237	4,271	3,634	+ 590	63.33	6.51	+15.06
26	73.49	50 mg. daily	1,090	5,195	− 210	3,976	3,487	+1,032	61.36	6.51	+17.03
27	73.92		1,146	5,240	− 311	3,945	3,474	+1,076	58.18	6.51	+20.21
28	74 71	50 mg daily + 9 alpha F 125 µg. daily	1,173	5,200	− 298	4,267	3,390	+ 838	62.64	6.51	+15.75
29	75.32		1,618	5,255	− 798	4,032	3,364	+1,099	51.90	6.51	+26.49
30	76.97		2,080	5,365	−1,370	4,317	3,405	+ 773	57.18	6.51	+21.21



31	77.51	37.5 mg daily	2,523	5,355	−1,803	4,466	3,418	+ 611	50.85	6.51	+27.55
32	78.50		2,580	5,415	−1,920	5,333	3,427	− 265	59.80	6.51	+18.49
33	78.22		2,775	5,320	−2,020	5,283	3,362	− 150	62.40	6 51	+15.99
34	78 37		2,839	5,455	−2,219	5,765	3,462	− 732	63.85	6.51	+14.54
35	78.25		2,647	5,230	−1,802	5,437	3,366	− 308	63.19	6.51	+15.20
36	78.00		2,540	5,265	−1,730	5,540	3,412	− 457	66.88	6.51	+11.51
37	78.18		2,267	5,110	−1,302	5,593	3,330	− 428	67.28	6.51	+11.11
38	77.24		2,039	5,015	− 979	5,649	3,319	− 473	70.68	6.51	+ 7.71
39 (7 days)	77 80		3,111	6,545	−1,151	7,252	3,217	+1,424	67.65	6.51	+ 9.92
40	76.98		2,131	4,765	− 821	4,675	3,386	+ 434	61.80	6.51	+16.59
41	77.30	75 mg. daily	1,646	4,625	− 196	5,008	3,414	+ 73	67.88	6.51	+10.51
42	77.18		1,465	4,530	+ 80	5,272	3,428	− 205	70.89	6.51	+ 7.50
43	76 69		1,482	4,650	− 57	5,078	3,524	− 107	71.75	6.51	+ 6.64
44	76 63		1,244	4,505	+ 326	5,105	3,383	+ 7	73.60	6.51	+ 4.79
45	76.34	75 mg. daily; 9 alpha F omitted	899	4,715	+ 461	4,821	3,470	+ 204	75.54	6.51	+ 2.85
46	75.34		562	4,545	+ 968	4,875	3,309	+ 311	75.95	6.51	+ 2.44
47	74.96		444	4,630	+1,001	4,868	3,377	+ 250	79.59	6.51	− 1.20
48	74.63	75 mg. daily + 9 alpha F 125 μg. daily	458	4,550	+1,067	4,921	3,346	+ 228	75.28	6.51	+ 3.11
49	75.56		725	4,585	+ 765	4,585	3,376	+ 534	71.56	6.51	+ 6.83
50	75.73		831	4,297	+ 947	4,442	3,194	+ 859	72.93	6.51	+ 5.46
51	75.82	37.5 mg. daily	774	4,605	+ 696	4,917	3,320	+ 258	74.58	6.51	+ 3.81
52	75.38		776	4,392	+ 907	5,066	3,108	+ 321	75.13	6.51	+ 3.26
53	75.11		838	4,255	+ 982	4,719	2,943	+ 833	74.47	6.51	+ 3.92
54	75.35		874	4,303	+ 898	4,853	3,016	+ 626	71.81	6.51	+ 6.58
55	75.33	Cortisone omitted	1,072	4,261	+ 742	5,145	2,985	+ 365	72.37	6.51	+ 6.02
*56	75.62	Cortisone 25 mg. daily	883	4,005	+ 268	4,317	2,897	− 811	65.52	6.51	−11.34
57	73.52		841	4,005	+1,229	5,100	2,844	+ 551	77.05	6.51	+ 1.34
58	74.37		977	3,999	+1,094	4,723	2,920	+ 852	67.92	6.51	+10.47
59	74.81		928	3,980	+1,167	4,840	2,769	+ 886	70.03	6.51	+ 8.36
60	74.63		903	3,921	+1,251	5,193	2,709	+ 593	70.70	6.51	+ 7.69
61	74.67		907	3,880	+1,288	5,201	2,668	+ 626	68.71	6.51	+ 9.68
62	73.14		912	3,880	+1,283	5,210	2,668	+ 617	72.05	6.51	+ 6.34
63	73.53		847	3,880	+1,348	5,227	2,668	+ 600	72.03	6.51	+ 6.36
	74.10										

Mean Intakes	*Calcium*	*Phosphorus*	*Nitrogen*
Periods 1- 7	5,734 mg.	8,036 mg.	83.11 Gm.
8-19	5,982 mg.	8,482 mg.	83.11 Gm.
20-63	6,075 mg.	8,495 mg.	84.90 Gm.

* Corrected for diet reject and emesis

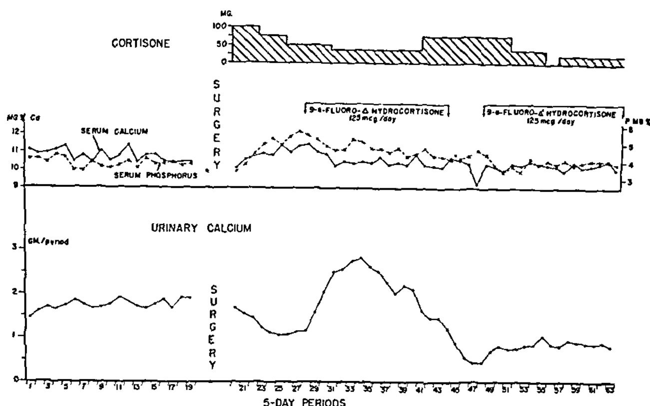


Fig. 3. Effect of adrenalectomy and replacement therapy on excretion of calcium in urine per period. Note high urinary excretion of calcium preoperatively with essentially normal values for serum calcium and inorganic phosphorus; postoperative decrease in calcium in urine and increase in serum inorganic phosphorus (Periods 20-25). Marked increase in urinary calcium associated with decrease in cortisone replacement therapy to 50 mg daily (Periods 26-41). The hypercalciuric episode appeared to be self-limited and could not be reproduced (Periods 51-63).

H.S tended to be somewhat overweight, and his caloric intake was set at 2,128 calories: protein, 129 Gm.; fat, 89 Gm , carbohydrate, 212 Gm. Although he lost weight slowly, except as the weight was influenced by specific medication (i.e., 19-nortestosterone cyclopentylpropionate), the caloric value of the food appeared to be adequate to maintain nitrogen balance. No significant modifications in the daily menu were required during the 225 days devoted to this phase of his study. However, analysis of the food indicated some reduction in intake of nitrogen, calcium and phosphorus, which occurred in Period 26 This appeared to be related to the source of skim milk used in the diet. The mean daily values were. Periods 1 through 25—for nitrogen, 21.10 Gm.; calcium, 1,130 mg.; phosphorus, 1,914 mg. Periods 26 through 45—nitrogen, 20.14 Gm.; calcium, 1,000 mg.; phosphorus, 1,831 mg. (Table 1). The balance data are recorded in Figures 7 to 9, 11 and 12 and in Table 3.

RESULTS

D.M.

Before Adrenalectomy. During the 96 days of preoperative study the balances of calcium and phosphorus were negative despite high intakes of these elements. While the losses were not as pronounced as have been observed in more advanced cases,[19,44,34,4] nevertheless, when considered in relation

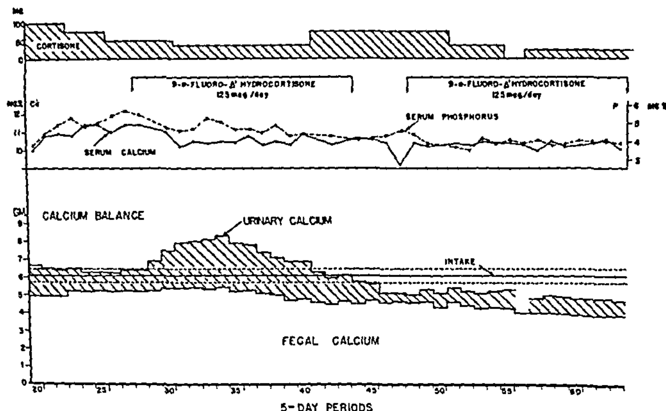


FIG. 4. Postoperative calcium balance. Graph similar to Figure 1. Note that fecal calcium has been plotted as a 5-period moving average to demonstrate tendency of calcium in stool to decrease as recovery progressed.

to the clinical picture, they strengthened somewhat the diagnosis of hyperadrenocorticism. Nitrogen balance was positive to the extent of 44.7 Gm. corrected to 6.30 on the basis of the daily loss of 0.4 Gm. from skin for the 19 periods.[41] Concentrations of serum calcium and inorganic phosphorus were not especially remarkable except that calcium on several occasions approached the upper limits of normal for the laboratory. The excretion of calcium in the urine was high and averaged 350 mg in 24 hours (Fig. 3 & Table 2). Although this is not an exceptionally high value, it exceeds the amount found in the urine of most normal subjects ingesting a comparable amount of calcium.[26,38] Applying the formula suggested by Malm,*[38 (p 178)] the net absorption of calcium was found to be 26 per cent and absolute absorption 55 per cent. Both percentages are somewhat lower than Malm's means for men ingesting 900 to 1,000 mg. of calcium a day but are still well within the range of values reported for his series. As D.M. received an average of 1,177 mg. of calcium daily, our results, while not strictly comparable, suggest nevertheless that if there was any depletion of osseous mineral reserves, it was accomplished through increased renal loss of calcium rather than an absorption defect. Balance studies were necessarily discontinued during the 8 days that were required for adrenalectomy and immediate postoperative recovery.

After Adrenalectomy. After removal of the adrenals, adjustment to a maintenance dose of cortisone was made slowly to permit evaluation of the effect of the medication on the balance. At initial doses of 100, then

* Per cent net absorption $= \frac{(\text{Ca Intake} - \text{Ca Feces})}{\text{Ca Intake}} \times 100$

decreasing to 75, mg. of cortisone daily, no particular differences were noted in the apparent well-being of the patient.

Serum Calcium and Phosphorus. Shortly after return to the standard diet, the level of serum inorganic phosphorus rose slowly and reached a maximum of 5.7 mg. % in Period 27 (Fig. 3). This occurred subsequent to reduction in the dose of cortisone to 50 mg. daily. Some elevation of phosphorus then persisted through the 46th period. The serum calcium (Fig. 3) level rose also in the early part of the study and remained in the high normal range through Periods 24 to 29.

Calcium in Urine. Urinary calcium, on the contrary, decreased slowly until Period 26, when the average 24-hour excretion was 218 mg. (Fig. 3). Mild symptoms of malaise, anorexia and aching joints, which at first were thought to be caused by adrenal insufficiency, became apparent in Period 28 and led to the incorporation of 9 alpha F in the regimen. There followed a prompt gain in weight of about 2 Kg. (Fig. 5) and a considerable increase in urinary calcium (Figs. 3 & 4). Further reduction in the dose of cortisone to 37.5 mg. daily—Period 31—intensified the patient's complaints. However, there was no fall in blood pressure or evidence of collapse. About 20 days later the calcium excreted in urine reached a

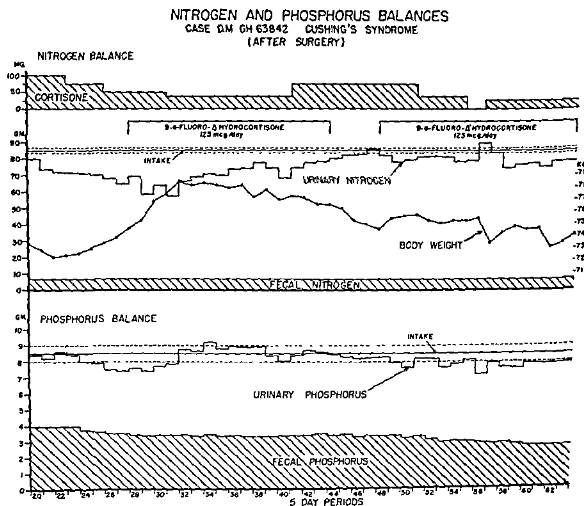


Fig. 5. Postoperative nitrogen and phosphorus balances Graph prepared as in Figure 1. Fecal phosphorus plotted as 5-period moving average. Note that considerable retention of nitrogen occurs prior to and during the marked negative calcium balance shown in Figures 4 and 6.

maximum of 568 mg. in 24 hours. Thereafter, although the same dose of glucocorticoid was continued, the urinary calcium began to diminish steadily. Resumption of the daily dose of 75 mg. cortisone in Period 41 brought about marked relief from unpleasant symptoms and a general improvement in morale. The gradual decrease in urinary calcium continued to a low point of 88 mg. in Period 27. Since the hypercalciuric episode had coincided with the administration of 9 alpha F, and the minimal calciuria had occurred during Period 47 after this medication had been omitted for 10 days, an attempt was made to reproduce the condition leading to hypercalciuria.

It was thought that mild glucocorticoid deficiency in the presence of an adequate dose of 9 alpha F might be the determining factor. The salt-regulating steroid was given again in the same dose as previously (125 mcg. daily). In 5 days the urine calcium increased and became stabilized at approximately 160 mg. daily. The daily cortisone then was reduced from 75 to 37.5 mg., with no significant change in calciuria. Cortisone finally was omitted for 6 days (Period 55 and the 1st day of Period 56), during which the patient became moderately ill. The peak value for urinary calcium at this time was 250 mg. daily.

During the final periods of the balance

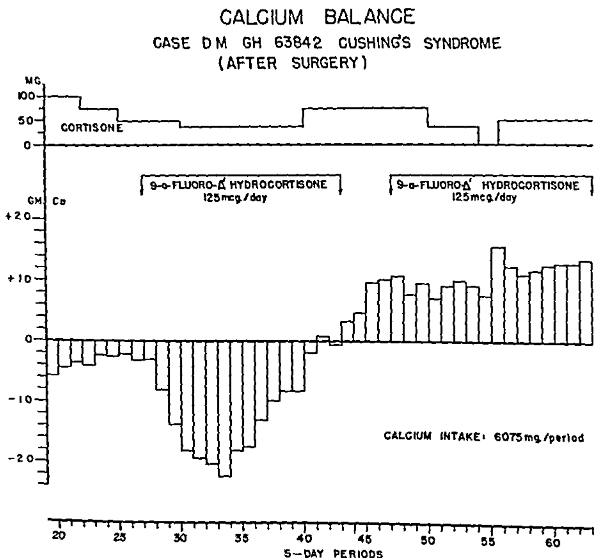
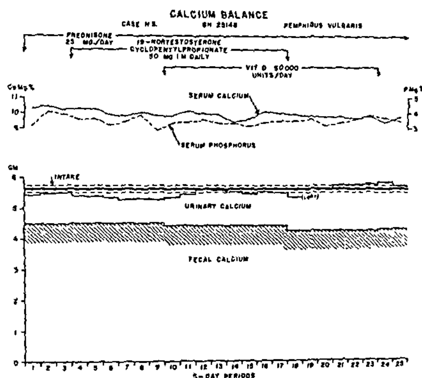


FIG. 6. Postoperative calcium balance Gains and losses of calcium plotted for each 5-day period. Scale approximately 3 times that employed in preparation of Figure 4.



Figs. 7 to 12, Case H.S. Fig. 7. Part 1. Calcium balance. Graph prepared as in Figure 1. Note that urinary calcium is shown as a clear area above fecal excretion. Several periods have been combined to obtain mean values used in plotting calcium in feces. See Table 3 for periods averaged.

study 25 mg. of cortisone was given daily—12.5 mg. morning and evening. The patient felt comfortable, was up and about the ward, and calcium in the urine ranged between 160 and 190 mg. daily. Repetition of the earlier hypercalciuric phenomenon was not observed.

Calcium Balance. The excretion of calcium exceeded the intakes in Periods 20 through 41 (Fig. 4 & Table 2). As fecal calcium remained relatively constant during these 105 days, the negative balance paralleled the urinary calcium closely. Subsequent to Period 41 there appeared to be a gradual decrease in calcium excreted in the stools. In order to reveal this trend, a 5-period moving average has been used in tabulating and plotting fecal values (Fig. 4). That fecal as well as urinary calcium was high in the first 100 days of recovery is indicated by calculation of net absorption, which was found to be 11.5 per cent as against 26 per cent preoperatively. During the final 50 days of balance, while calcium was being retained in appreciable amounts, net absorption had increased to 34 per cent. The following tabulation is a summary of calcium exchange:

Preoperative and Postoperative Calcium Balance

Preoperative periods 1-19 (95 days)	− 4.0 Gm.
Postoperative periods 20-41 (110 days)	−20 7 Gm.
Postoperative periods 42-63 (110 days)	+18.7 Gm
Net Balance	− 6 0 Gm

Nitrogen and Phosphorus Balances. Considerable storage of nitrogen was noted after return of the patient for the postoperative study (Fig. 5 & Table 2). This contrasted sharply with the calcium balance, which became most strongly negative (Fig. 6) at the time that nitrogen retention was greatest.

Phosphorus exchange, which is influenced by both nitrogen and calcium,[50] was negative in Periods 32 through 38 and again in Periods 42 and 43. As in the case of fecal calcium, a 5-period moving average has been employed to demonstrate the tendency for phosphorus excretion in the stools to decrease. Calculation of a "theoretical phosphorus balance"[50] indicated that the estimated retention of phosphorus was considerably greater than the actual. Correction of the observed nitrogen balance by a factor

of 0.4 Gm. daily presumably lost from the skin[41] reveals the following relationship:

DAYS OF BALANCE 222

Nitrogen balance + 343 Gm.
Phosphorus equivalent of retained nitrogen .... + 23.30 Gm.
Calcium balance − 3.03 Gm.
Phosphorus equivalent of calcium loss .... − 1.36 Gm.
"Theoretical" phosphorus balance .... + 21.94 Gm.
Observed phosphorus balance .. + 14.35 Gm.

Only about two thirds as much phosphorus was retained as the balances of calcium and nitrogen appeared to require. While no explanation of this divergence is immediately apparent, the possibilities of small systematic errors in the various procedures and analyses

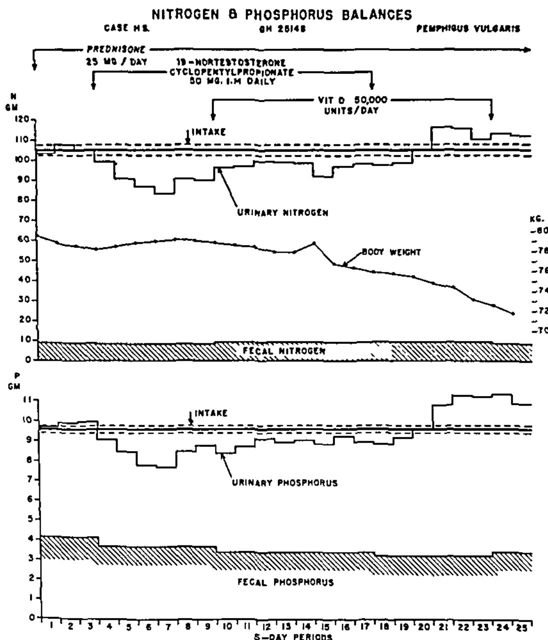


FIG. 8. Part 1 Nitrogen and phosphorus balances Graph prepared as in Figure 1. Note that Fecal Excretion Periods 1 to 3, 4 to 9, 10 to 17, 18 to 23 and 24 to 25 have been averaged and are represented as hatched areas. Administration of androgen produced a well-defined anabolic response. Vitamin D seems to have caused a small decrease in fecal phosphorus. The nitrogen and phosphorus "rebound phenomenon" occurs after withdrawal of nortestosterone and in the case of phosphorus is exaggerated by continued administration of vitamin D.

are numerous. These could easily account for the results obtained.

H.S.

The balance data on this patient have been divided into two parts. Part 1, Periods 1 through 25 (Table 3 & Figs. 7-9), deals with his response to androgen and vitamin D while he was receiving a constant daily dose of prednisone of 25 mg. Part 2 is concerned primarily with an attempt to discontinue prednisone. Throughout the 225 days devoted to the study, his general condition remained satisfactory except for a brief febrile episode related to an acute urinary tract infection in Period 23, and some malaise, joint pains and nausea in Periods 39 to 42 attributable to the reduction in steroid therapy. Most of the time he was up and about the ward, and was even allowed to take short excursions from the hospital.

Part 1: Calcium Balance. During the first 3 periods that served as a control, the calcium balance was slightly positive, and urinary calcium averaged 195 mg. daily. Injections of 19-nortestosterone cyclopentylpropionate, 50 mg. daily in sesame oil, served to bring about a small decrease in renal excretion of calcium, which was about 40 mg. a day less than noted in the control periods (Table 3). This effect became clearly apparent after about 15 days. Addition of 50,000 I.U. vitamin D to the regimen (Period 10) produced a rather prompt in-

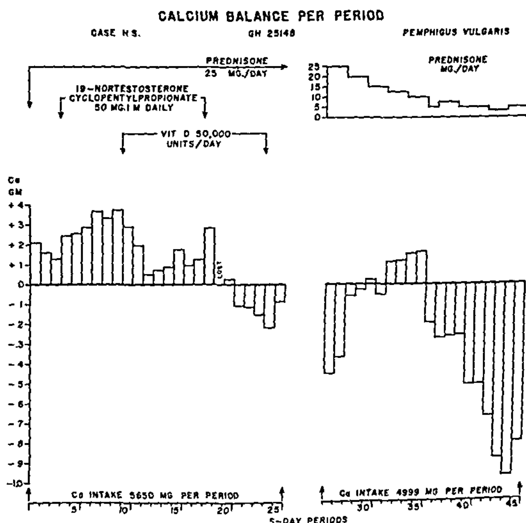


Fig. 9. Parts 1 and 2 Directional changes in calcium balance Scale approximately 5 times that employed in preparation of Figure 7.

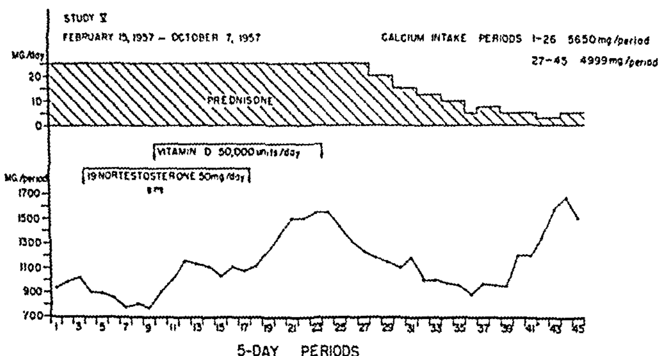


Fig. 10. Parts 1 and 2. Calcium excretion in urine per period. Note initial decrease in urinary calcium when nortestosterone was administered, followed by moderate increase when vitamin D was given and hypercalciuric effect of D when androgen was discontinued. Tapering off the dose of prednisone led to symptoms of "steroid withdrawal" (Periods 39-45), during which hypercalciuria also occurred.

crease in urinary calcium to about 220 mg. daily (Fig. 10). Androgen was discontinued at the end of Period 17, and, as the effect wore off, the urinary calcium rose under the influence of vitamin D to a maximum of 310 mg. (Period 24). Hypercalcemia did not develop. Net absorption of calcium was 20 per cent during control periods and remained the same during the first 6 periods on androgen. In the following 8 periods (10 through 17), when both androgen and vitamin D were given, there was little change. However, the continued administration of D (Periods 18 through 23) seems to have reduced the excretion of fecal calcium. Net calcium absorption was 24 per cent in these periods. Effects of medication are depicted in Figures 7 to 10. It is clear that vitamin D at this level exerted an unfavorable influence on calcium balance (Fig. 9).

Nitrogen and Phosphorus Balances (Fig. 8 & Table 3). Approximate nitrogen equilibrium in the control periods was followed by marked nitrogen retention, reaching its maximum between the 16th and the 20th days of androgen administration. Thereafter, while less nitrogen was stored, the balance remained positive until about 10 days subsequent to the discontinuance of nortestosterone. An initial, slightly negative phosphorus balance also became positive at this time and followed the exchanges of nitrogen rather closely. When the anabolic effects disappeared, the usual "rebound phenomenon" was noted for both nitrogen and phosphorus. One also observes a small decrease in fecal phosphorus, which appeared to be due to androgen, and a further decrease associated with administration of vitamin D. As in the case of calcium, there was an increase in urinary phosphorus while the patient was under the influence of the vitamin, and this in turn led to a distinctly negative balance.

Part 2: Calcium Balance (Fig. 11). As mentioned previously, Period 26 marks a small decrease in the intakes of calcium,

TABLE 3. CALCIUM, PHOSPHORUS AND NITROGEN BALANCES. SUBJECT H.S. HEIGHT 165 CM.

Period (5 Days)	Body Wt Kg.	Medication	Calcium mg. Urine mg.	Calcium mg. Stool mg.	Calcium mg. Balance mg.	Phosphorus mg. Urine mg.	Phosphorus mg. Stool mg.	Phosphorus mg. Balance mg.	Nitrogen Gm. Urine Gm.	Nitrogen Gm. Stool Gm.	Nitrogen Gm. Balance Gm.
1	79 51	*Prednisone 25 mg. daily	936	4,503	+211	5,408	4,132	+ 30	94.47	8.82	+ 2.21
2	78 76		984		+163	5,778		− 340	98.92		− 2.24
3	78 50		1,017		+130	5,851		− 413	96.13		+ 0.55
4	78.19	†Depo-nortestonate 50 mg daily I.M.	900	4,505	+245	5,381	3,690	+ 499	90.47		+ 6.21
5	78.33		890		+255	4,783		+1,097	81.98		+14.70
6	78.70		858		+287	4,096		+1,784	78.25		+18.43
7	78.95		779		+366	4,005		+1,875	74.32		+22.36
8	79.12		813		+332	4,769		+1,111	82.15		+14.53
9	79 06		774		+371	5,084		+ 796	81.47		+15.21
10	78 77	Vitamin D 50,000 units daily	913	4,448	+289	4,978	3,497	+1,095	86.93		+ 9.75
11	78.52		1,004		+198	5,296		+ 777	87.54		+ 9.14
12	78.35		1,156		+ 46	5,694		+ 379	90.17		+ 6.51
13	77.97		1,129		+ 73	5,524		+ 549	89.98		+ 6.70
14	77 87		1,113		+ 89	5,619		+ 454	89.29		+ 7.39
15	78.65		1,028		+174	5,449		+ 624	82 65		+14.03
16	76.64		1,105		+ 97	5,830		+ 243	87.67		+ 9.01
17	76 29		1,076		+126	5,527		+ 546	88.78		+ 7.90
18	75.92		1,102	4,269	+279	5,608	3,208	+ 754	88.25		+ 8.43
19	75.71		1,242		+139	5,963		+ 400	89.23		+ 7.45
20 (3 days)	75.46		816	2,561	+ 13	3,803	1,925	+ 14	57.53		+ 0.55
21	74.80		1,494	4,269	−113	7,577	3,208	−1,215	106.76		−10.08
22	74.35		1,500		−119	8,032		−1,670	106.36		− 9.68
23	73.13		1,539		−158	7,995		−1,633	101.89		− 5.18
24	72.52		1,552	4,227	−129	7,915	3,405	−1,750	104.46		− 7.78
25	71.72		1,424		− 1	7,446		−1,281	103.82		− 7.14
26	71 17		1,311		−539	6,585		− 835	94.52		− 2.64
27	70 78		1,229		−457	6,983		−1,233	95.71		− 3 83
28	70.51	20 mg daily	1,181	3,877	− 59	6,447	3,020	− 312	94.73		− 2.85
29	70.21	20 mg daily	1,149		− 27	6,211		− 76	94 40		− 2.52

30	69 96	15 mg daily	1,100		+ 22	5,750		+ 385	89.54		+ 2.34
31	69 81	15 mg daily	1,176		− 54	5,407		+ 728	88.04		+ 3.84
32	70.15	12 5 mg. daily	1,010		+112	5,258		+ 877	82.83		+ 9.05
33	70.36	12.5 mg. daily	1,007		+115	5,476		+ 659	83.03		+ 8.85
34	70 45	10 mg daily	969		+153	5,286		+ 849	78.54		+13.34
35	70 81	10 mg daily	960		+162	5,070		+1,065	75.34		+16.54
36	71.09	5 mg daily	887	4,307	−195	4,952	3,063	+1,140	77.31		+14.57
37	71 38	‡7.5 mg. daily	966		−274	5,271		+ 821	77.65		+14.23
38	71.58	7 5 mg daily	956		−264	5,497		+ 595	80.38		+11.50
39	71.43	5 mg daily	949		−257	5,538		+ 554	76.16		+15.72
40	72 16	5 mg daily	1,202		−510	5,365		+ 727	80.08		+11.80
41	72 10	5 mg daily	1,201		−509	5,769		+ 323	83.91		+ 7.97
42	72.29	3 mg. daily	1,364		−672	5,374		+ 718	80 42		+11.46
43	72.83	3 mg daily	1,577		−885	5,748		+ 344	72.81		+19.07
44	72.78	§5 mg daily	1,669		−977	5,904		+ 188	72.25		+19.63
45	71 96	5 mg. daily	1,495		−803	6,382		− 200	88.88		+ 3.00
	71 98										

Mean Intakes	*Calcium*	*Phosphorus*	*Nitrogen*
Periods 1-25	5,650 mg	9,570 mg.	105.50 Gm.
26-45	4,999 mg.	9,155 mg.	100.70 Gm.

* Δ^{1-4} cortisone.
† 19-nortestosterone 17β-cyclopentylpropionate.
‡ Demerol required in Period 37 and in Periods 39 through 44 for relief of joint pains incident to reducing dose of prednisone.
§ Dose of prednisone increased from 3 to 5 mg. per day on third day of Period 44.

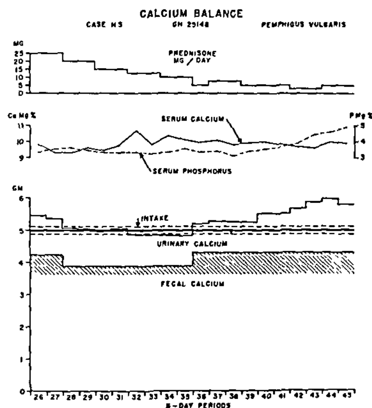


FIG. 11. Part 2. Calcium balance. Graph prepared as in Figure 1. Note rise in serum phosphorus, increased urinary calcium and negative calcium balance associated with reduction in dosage of prednisone (Periods 39-45).

phosphorus and nitrogen that occurred as a result of a change in the source of milk. Both the amounts of calcium and phosphorus in the diet were found to be lower by about 130 mg. daily, nitrogen by approximately 0.9 Gm. While it is doubtful, in view of the high intake, that urinary calcium could have been influenced noticeably by this small change, the gradual decrease in the amount excreted in each period seems to reflect decay in the action of vitamin D. Return approximately to control values was observed in Period 33, or 45 days after medication when the vitamin was stopped and while very slow reduction in the dose of prednisone was in progress (Fig. 10 & Table 3). It was hoped that any adrenal insufficiency secondary to prolonged corticosteroid therapy could be avoided by this maneuver. The condition of the patient remained satisfactory while the dose of prednisone was being lowered from 25 mg. to 5 mg. over an interval of 40 days Unfortunately, after 3 days at 5 mg. (Period 26), he complained of myalgia, arthralgia, marked weakness, headache and loss of appetite, and it became necessary to increase the dose of corticosteroid to 7.5 mg. for 10 days. A second, more successful, attempt to maintain the patient at the 5-mg. level of prednisone then was made. After 5 days the urinary calcium increased abruptly (Fig. 10), and, with further reduction in the dose to 3 mg., calciuria rose to 330 mg. daily. Symptoms previously noted again supervened but regressed when the prednisone was increased to 5 mg. Not only was the renal excretion of calcium greatly increased, but the net absorption also declined to 12 per cent in the last 5 periods. Therefore, both the gastro-intestinal tract and the kidneys contributed to the negative calcium balance (Fig. 9). The concentration of calcium in serum remained virtually unchanged; however, a small increase in the serum inorganic phosphorus was found in Periods 41 to 45. Because of the tedium of the balance program and since the patient felt well while taking a greatly reduced dose of corticosteroid, he requested discharge as an inpatient Spot checks of the 24-hour urine calcium were continued and revealed

a decrease to about 200 mg. at the end of a month.

Nitrogen and Phosphorus Balances (Fig. 12). The effect of prednisone on nitrogen exchange did not become obvious until the attempt to discontinue the drug (Period 28 *et seq.*) was under way. The balance became positive when the daily dose of corticosteroid was reduced to 15 mg. and was maximal when 3 to 5 mg. was given. A considerable increase in urinary nitrogen in the final period (45) suggested establish-

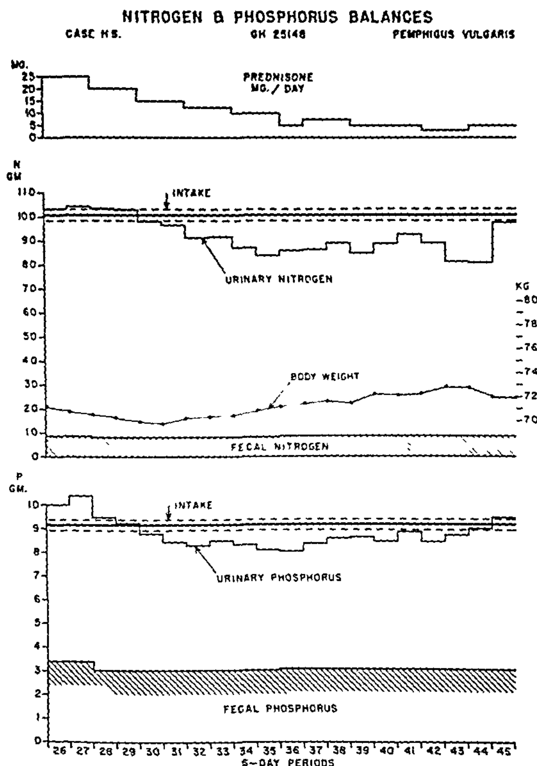


FIG. 12. Part 2. Nitrogen and phosphorus balances. Graph prepared as in Figure 1. Note positive balances associated with reduction in the dosage of prednisone.

ment of a new level of equilibrium. Unfortunately, this could not be confirmed because of the patient's decision to leave the hospital. The phosphorus balance followed that of nitrogen rather closely with maximum retentions in Periods 35 to 36 just prior to the rise in urinary calcium and development of negative calcium balance.

During the first 20 periods (Part 1), when a strongly positive nitrogen balance was induced by administration of an anabolic steroid, the agreement between actual phosphorus balance, +12.3 Gm., and "theoretical phosphorus balance," +11.2 Gm., was considered to be satisfactory. In the last 16 periods (30-45) there was a marked retention of nitrogen also, which seems clearly to have been the result of reducing the dose of prednisone. The actual phosphorus balance revealed a retention of 10.36 Gm. while the "theoretical phosphorus balance" was 8.1 Gm. In Periods 21 through 29 in which the rebound phenomenon occurred following discontinuance of androgen and when the calcium balance became negative due to the hypercalciuria of vitamin D administration, more phosphorus was lost than could be accounted for. The actual balance showed a loss of 10.00 Gm., while the indicated loss based on negative balances of calcium and nitrogen was only 5.6 Gm.

DISCUSSION

Effect of Vitamin D During Treatment With Prednisone and an Anabolic Steroid

The employment of corticosteroids for their "normalizing" effect in hypercalcemic states stems from the observation that the elevated levels of serum calcium observed occasionally in sarcoidosis respond promptly to steroid therapy[54,15,6,24,31] It now has been clearly established that the hypercalcemia caused by hypervitaminosis D can be controlled by the administration of adrenocorticotropin and cortisone and analogues of the latter.[13,61] Although accumulating evidence suggests that the site or sites of action of glucocorticoids and vitamin D differ[23,14,58,59] and that one steroid does not directly antagonize the effect of the other, the fact that large doses of vitamin D tend to diminish the fecal excretion of calcium while large doses of cortisone sometimes have the opposite effect[19,34,56,35,1] is no doubt one of the reasons for employing the vitamin in attempts to achieve calcium equilibrium in hyperadrenocorticism.

This reasoning overlooks the fact that the osseous disease in cushingoid states is that of osteoporosis[2] rather than osteomalacia and that the primary defect is more likely one of failure to form adequate amounts of protein matrix in bone. Thus, even if better absorption were obtained, improved mineralization of bone would not occur due to lack of structural material on which bone salts can be deposited. Whether or not vitamin D can act as a stimulus to osteoid production in adult humans in unknown, although there is evidence that this may be the case when toxic doses are administered to weanling rats.[58] However, the ability to form osteoid in hypervitaminosis D does not prevent simultaneous resorption and demineralization of bone.[53,22,43 52,7]

The use of an anabolic steroid to counteract the protein depleting and demineralizing effects of cortisone and some of its newer synthetic analogues has been proposed.[48,49,23] In the experience of the authors, some clinicians favor combining this treatment with the administration of rather excessive doses of vitamin D, often overlooking earlier work referring specifically to the fact that neither a protective nor a therapeutic action of the vitamin has been demonstrated in Cushing's syndrome.[19,44,3]

To summarize the phase of the study of H.S. in which prednisone, 19-nortestosterone cyclopentylpropionate and vitamin D were given in the combination indicated in Figures 7 to 10 and Table 3, the balances reveal (a) a strong anabolic effect of nortestosterone as measured by nitrogen and

phosphorus retention; (b) a small favorable effect on calcium balance *resulting from* decrease in urinary calcium due to androgen; (c) a loss of this advantage by the addition of 50,000 units daily of vitamin D to the program but apparent partial control of hypercalciuria by the androgen; (d) a considerable increase in urinary calcium and possibly a small decrease in fecal calcium when androgen was omitted and prednisone and vitamin D were given together. The net effect of the vitamin on calcium balance was unfavorable and certainly did not recommend it as an antagonist to prednisone. While hypercalcemia did not develop during administration of the vitamin to this patient, it must be admitted that, owing to inability to discontinue corticosteroid therapy because of his *skin lesions, it was impossible to ascertain* whether or not prednisone prevented the hypercalcemia of D toxicity. The increased excretion of calcium in the urine[8,47] may well be a more sensitive indicator of hypervitaminosis D in man than is hypercalcemia, and, in the present case, this action of the vitamin did not appear to be affected by the dose of glucocorticoid that he received.

Hypercalciuria During Corticosteroid Withdrawal

The suppression of adrenocortical function after prolonged administration of glucocorticoids is well recognized.[11,9] When therapy is to be discontinued or reduced markedly, gradual reduction in dosage is indicated in order that the gland may have an opportunity to recover and resume a normal hormonal output. Withdrawal symptoms usually are attributed to hypoadrenalism, and doubtless this is a reasonable explanation, especially when fever, hypotension, signs of shock and collapse supervene

Both our patients were at first thought to manifest mild symptoms of adrenal insufficiency at the time that their daily dose of steroid had been reduced substantially. However, in each case, after a week or more of complaints of anorexia, moderate malaise, *myalgia and joint pains, adaptation to the* lower dose of cortisone (D.M.) or prednisone (H.S.) occurred. Thereafter, doses previously associated with these symptoms appeared to be adequate to maintain them in a condition of health.

Rather similar symptoms have been commented upon following subtotal adrenalectomy for Cushing's syndrome. According to Kepler *et al.*,[31] in four properly prepared patients

> after partial removal of the second adrenal gland . . . convalescence seemed to proceed uneventfully. Sometime during the first week or shortly thereafter the patients complained of nausea. They lost their appetites and vomited. The anorexia became more and more intense until finally they refused all food. Even the sight of food became repulsive. About the same time abdominal pain and tenderness made their appearance. Gradually the chemical composition of the blood became disordered. This disturbance was generally characterized, when the patient was not being treated with adrenal cortical extract or electrolytes, by low concentrations of both sodium and potassium. The values for blood urea, and as time went on the concentration of blood calcium, slowly tended to rise and eventually attained levels seen in hyperparathyroidism. Phosphate levels, however, were not depressed.

The maximum concentration of serum calcium, as reported by Kepler, was of the order of 14 mg. %. This strongly suggests a disturbance in calcium homeostasis in patients recovering from prolonged exposure to the effects of excessive amounts of glucocorticoids. Indeed, at times, anorexia, vomiting and abdominal pain are manifestations of hypercalcemia.[28,60] Unfortunately, the excretion of calcium in the urine was not measured. That the symptom complex in Kepler's patients may not have been entirely explicable on the basis of adrenal insufficiency may be inferred also by the fall in concentration of serum potassium and their failure to become hypotensive.

The events leading to hypercalciuria in our subjects were not associated with hyper-

calcemia, perhaps because they were not as ill as Kepler's patients. While our cases showed malaise, joint pains and considerable anorexia, neither of them developed hypotension, fever or signs of collapse. The symptom complex can perhaps be described as "the steroid withdrawal syndrome,"[5] and, according to Bondy *et al.*,[10] in such individuals there is no lack of circulating Cortisol during the withdrawal period, when symptoms are maximal. The balance data indicate a strong phase of anabolism manifested by marked retention of phosphorus and nitrogen and a tendency of the serum inorganic phosphorus to rise. Simultaneously, hypercalciuria with negative calcium balance occurred. These are precisely the findings that have been noted where human growth hormone has been administered in what appears to have been somewhat excessive amounts.[12,29] It seems significant, too, that in the patients followed by Ikkos, Luft and Gemzell[29] fairly large doses of human growth hormone produced symptoms similar to those that we have observed. Thus, a possible explanation of the alterations in the balances is the release of more than physiologic amounts of growth hormone.

While prolonged treatment with large quantities of adrenocorticosteroids may lead to depletion of ACTH or malfunction of the pituitary adrenal system,[27] Ingbar and Freinkel[30] have discussed the possibility that, in the rat, at least in the case of thyroid-stimulating hormone, prolonged exposure to large doses of cortisone may produce compensatory overactivity of the pituitary with increased secretion of thyrotropin. Should this compensation extend to growth hormone also, one might suppose that in our subjects, both of whom had been exposed to excessive amounts of glucocorticoids over long periods, an attempt at compensation by the pituitary had occurred. When antianabolic effects of corticoids were removed, each man was left temporarily with an excess of growth hormone. The latter produced nitrogen and phosphorus retention and at the same time led to hypercalciuria Growth hormone also has been reported to cause a rise in the serum inorganic phosphorus, which agrees with our findings.

While we have no proof that this is what actually took place, it seems a less complicated and more reasonable solution than could be arrived at by assuming changes in parathyroid function.

Remineralization of Bone After Cure of Cushing's Syndrome

The present study does not contribute much to the problem of the extent of bone remineralization. D.M., who showed little skeletal rarefaction, eventually achieved a strongly positive calcium and phosphorus balance after his adrenals were removed (Periods 44-63). However, during the total time of measured calcium exchange he still demonstrated a small net loss of this element. H.S., who showed some roentgenographic evidence of bone atrophy, never could be rehabilitated completely and always required moderate to large doses of corticosteroid. While retentions of calcium were observed from time to time, these were at least equaled by intervals of negative balance. That partial remineralization of bone can occur following spontaneous or operative cure of Cushing's syndrome is known;[55,20] but whether the bone disease is completely curable is still a debatable issue.

SUMMARY

The exchanges of calcium, phosphorus and nitrogen have been studied in two patients, in one of whom adrenalectomy was performed for spontaneously arising Cushing's syndrome. In the second subject, cushingoid features developed as a result of prolonged corticoid administration for pemphigus vulgaris. Androgen and vitamin D were given to the latter to observe whether or not either agent opposed the demineralizing effects of glucocorticoids. Both men were studied also during partial withdrawal of steroid therapy. The metabolic data obtained indicated that

1. when compared with intake, the ab-

sorption of calcium tended to be somewhat low in both subjects but was still within normal limits;

2. urinary calcium was high in the patient with spontaneously arising Cushing's syndrome, while in the man whose cushingoid state was secondary to prolonged glucocorticoid administration, calciuria remained within the normal range;

3. anabolic steroid therapy tended to reduce urinary calcium excretion during treatment with prednisone (Case H.S.) and partially to control the hypercalciuria of excess vitamin D;

4. prednisone in doses of 25 mg. daily did not prevent 50,000 I.U. of vitamin D from inducing hypercalciuria and negative calcium balance, hence it is concluded that large doses of D, if anything, augment the demineralizing effects of prednisone;

5. "steroid withdrawal symptoms" occurred in both patients during reduction in dosage of glucocorticoid and were accompanied by hypercalciuria, negative calcium balance, retention of nitrogen and phosphorus, and a rise in the concentration of serum inorganic phosphorus. It is suggested that this syndrome results from temporary oversecretion of pituitary growth hormone.

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Studios Metabolic in Syndrome de Cushing; le Effecto que le Suppression de Steroide e le Administration de Androgeno e Vitamina D Exerce Super le Balancia de Calcium, Phosphoro e Nitrogeno

Summario in Interlingua

Le excambio de calcium, de phosphoro, e de nitrogeno esseva studiate in duo patientes. In un del duo, adrenalectomia esseva executate a causa de syndrome de Cushing de genese spontanee. In le secunde subjecto, aspectos cushingoide se disveloppava como resultato de un administration prolongate de corticoide in le tractamento de pemphigo

SECTION II

GENERAL ORTHOPAEDICS

31

Eosinophil Responses to ACTH in Relation To Bone Maturation in Crippled Children

PHILIP RADDING, M.D.*

This is a report of observations on the eosinophil responses to ACTH in relation to bone maturation in crippled children.

METHODS AND TECHNICS

Total circulating eosinophil counts were obtained fasting and 4 hours after the intramuscular injection of 25 units ACTH, using the chamber count technic. The diluent for performing the counts was prepared as follows:

1. Propylene glycol .... 50 ml.
2. Distilled water .... 40 ml.
3. Sodium carbonate 10% .... 1 ml
4. Phloxine 1% .... 10 ml

The consistency of responses to intramuscular ACTH on repeat testing appeared to be fairly good, as seen in Table 1. None of the Thorn tests was performed during the convalescent period of a recent surgical procedure, however, the eosinophil responses to minor surgical trauma were observed in three patients with Legg-Calvé-Perthes syndrome (LCPS) by obtaining a fasting preoperative count and periodic postoperative counts. The surgical procedures in all three were the same; i.e., the determination of the direct circulation of the femoral capital epiphyses This required the insertion of a special probe into the epiphysis under general anesthesia after administering radioactive phosphorus and, generally, lasted from one half to three quarters of an hour.

In all but 6 of the 81 patients tested by the Thorn method, roentgenograms of the hands were taken for bone age determinations in accordance with the standards of Greulich and Pyle.[9] Two completely separate and independent readings of bone age were made on each roentgenogram, using the average of the two if they were within 6 months of each other. If the difference were greater than 6 months, a third independent reading was made, and the average of any two that were within 6 months of each other was used as the bone age. In one instance, where the first two readings were 1 year apart and the third one fell halfway between, the median reading was used.

Full data tables on age, sex, eosinophil counts and bone age determinations are given in Tables 8 to 13, inclusive.

RESULTS

The results were divided into two major groups: (1) a group with Legg-Calvé-Perthes syndrome (LCPS) in which bone maturation is known to be disturbed; (2) a control group consisting of all remaining diagnoses in which a disturbance of bone maturation is not a clinical feature. In the former, there were 37 males and 10 females with a chronologic age range of 36 to 145 months and a mean of 85 months. In the latter, there were 16 males and 18 females with an age range of 41 to 185 months and a mean of 95 months.

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The Thorn test results are summarized statistically in Table 3. The control group had a mean fasting count of 257/mm.[3] with a spread of 150 to 375/mm.[3], as compared with a mean of 226/mm.[3] and a spread of 77 to 333/mm.[3] in the LCPS group. Equivalent standard deviations of the fasting counts and the percentage fall in count indicate that comparable methods and technics were used on both groups. From the average percentage fall in count, which was 51 per cent in the control group and 46 per cent in the LCPS group, it would appear that there were a significant number of less than 50 per cent responses in both groups. The question raised was whether this was due to chronicity of disease in both groups or to variations of potency and absorption

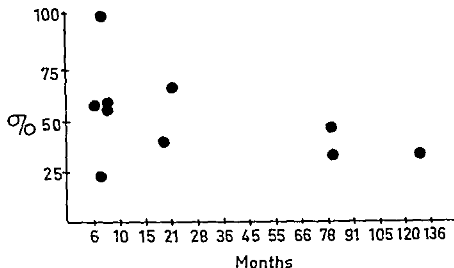


Figure 1

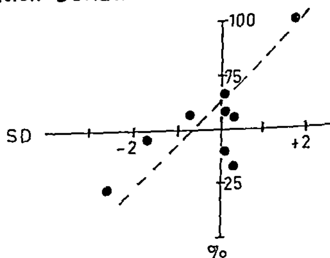
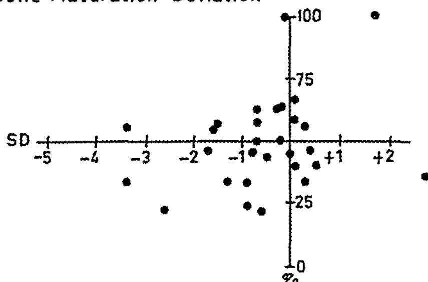


Figure 2

of the ACTH. Since the total series was accumulated gradually over several months by performing Thorn tests on a few patients at a time from both groups, these variables should be distributed equally and should be reflected in a similar distribution of the results in both groups if it were the latter. By breakdown of the results by frequency of occurrence at intervals of 10 per cent response, as shown in Table 3, 25.5 per cent of the LCPS group was found to have less than 30 per cent fall in eosinophils as compared with 8.8 per cent of the control group; while 66.0 per cent of the former and 85.2 per cent of the latter had results that fell into the 31 to 70 per cent response range. This represents a significant difference in the distribution of the results, indicating that po-

FIGURE 3



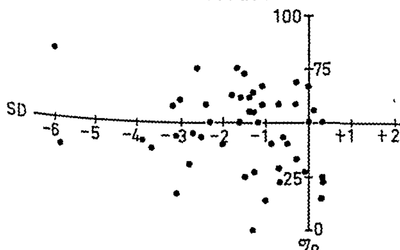


FIGURE 4

tency and absorption factors were less likely to account for the lack of responsiveness observed.

The influence of disease chronicity on adrenal responsiveness was difficult to assess separately from such factors as the nature and the severity of the disease process, exposure to trauma, etc., which may be operative simultaneously. The diagnostic diversity of the control group could be overcome partially by division into four general categories, as shown in Tables 4 to 7, inclusive. Of these, only the poliomyelitis category appeared to be suitable for analysis of the results in relation to disease chronicity, since the onset could be dated accurately. Graphic correlation of the eosinophil responses with elapsed time from onset of disease and bone maturation—Figures 1 and 2, respectively—showed some evidence of a relationship with both factors, each of which appeared *to be acting independently,* as they themselves were found to be unrelated graphically.

With respect to disease chronicity in the LCPS group, the insidious onset of symptoms, which do not necessarily parallel the roentgenographic appearance of the capital femoral epiphyses, obviates any possibility of dating the disease onset accurately. If the onset of symptoms is used as a criterion, then length of hospitalization in this series provides some index of chronicity, since most of the patients were referred for admission within a few weeks of the time that symptoms were first noted, when the diagnosis was confirmed by x-ray. When months of hospitalization, which also reflected the duration of treatment in supervised and controlled recumbency, were graphed against percentage eosinophil fall and bone maturation deviation, no evidence of any relationship was found. When bone maturation, which characteristically is retarded in this group, was graphed against Thorn test results (Fig. 4), the direct relationship indicated in Figure 2, which was confirmed for *the control group as a whole (Fig. 3),* appeared to be conspicuously absent. Because of the number of uncontrollable variables, plus the inherent inaccuracies of the methods, points distribution in Figures 3 and 4

Average Eosinophil Responses for Various Intervals of Bone Maturation

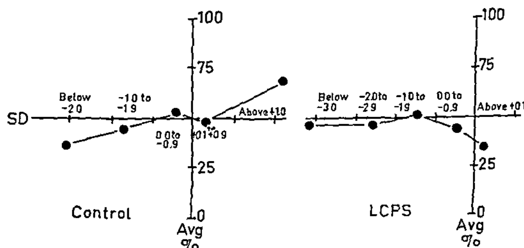


FIGURE 5

were fairly wide. To visualize more clearly the differences between the control and the LCPS groups, for each whole unit of bone maturation deviation the average eosinophil responses were plotted against the average maturation deviation, since the statistical study showed definite comparability of applied methods and technics. As seen in Figure 5, 4 out of the 5 points plotted in the control group fell along a straight line, substantiating strongly the evidence of a direct relationship between eosinophil responses and bone maturation. In the LCPS group in contrast, there was a tendency for the average responses to remain level, as skeletal maturation became less delayed with a dip in the average percentage fall in eosinophils as positive bone maturation was approached.

On the basis of this preliminary study, there would appear to be a need for further investigation of the relationship between adrenal function and bone maturation using quantitative steroid determinations for more accuracy in assessing adrenal function. The particular application of this information to the problem of retarded bone maturity in Legg-Calvé-Perthes syndrome is evident. From the few observations made on the eosinophil responses of LCPS patients to minor surgical trauma (Fig. 6), it was apparent that no adrenal exhaustion or insufficiency problem was involved. If anything, there would appear to be some tendency for the adrenals to overreact to the amount of trauma inflicted. Therefore, if any adrenal dysfunction does exist in this disease, it would probably be a relative change in activity of those factors that influence bone growth and maturity.

CONCLUSIONS

Observations are presented on the eosinophil responses to ACTH and bone maturation in crippled children. There appears to be a definite need for further investigation of adrenal function in relation to bone maturation. Such a study might provide information of significance to the problem of retarded skeletal maturation in Legg-Calvé-Perthes syndrome.

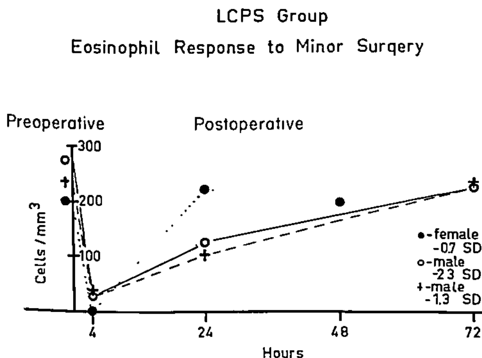


FIGURE 6

Table 1. Consistency of 4-Hour Eosinophil Responses to Intramuscular ACTH

Patient	Date of Test	Fasting Count	4-Hour Count	% Fall
IS	2-23-56	217	93	57.1
	4-10-56	250	100	60 0
AF	3- 5-56	248	93	62.5
	3-22-56	275	125	54.6
CC	3-27-56	217	93	57.1
	4-11-56	225	75	66.7
MA	3-27-56	186	0	100.0
	4-11-56	175	50	71.4
TC	1-22-58	244	244	0 0
	1-24-58	266	266	0.0

Table 2. Statistical Summary of Results of Fasting Eosinophil Counts and the 4-Hour Responses to Intramuscular ACTH

	Legg-Calvé-Perthes Syndrome	Control
Fasting Counts		
Mean	226	257
Standard deviation	56	51
Coefficient of variability	25.8	19.8
Percentage Eosinophil Fall		
Mean	46.0	51 0
Standard deviation	18 5	17.4
Coefficient of variability	40.2	34.1

Table 3 Thorn Test Results; Distribution of Results by Percentage Groups

	Control N = 34		LCPS N = 47	
	No	%	No.	%
0- 10%	0	0.0	1	2 1
11- 20%	0	0.0	3	6 4
21- 30%	3	8.8	8	17 0
31- 40%	8	23.5	6	12.8
41- 50%	8	23.5	10	21.3
51- 60%	9	26.5	8	17 0
61- 70%	4	11.8	7	14 9
71- 80%	0	0 0	3	6 4
81- 90%	0	0 0	1	2 1
91-100%	2	5.9	0	0.0

Table 4. Composition of Poliomyelitis Patients in Control Group in Relation to Bone Maturation and Thorn Test Results

Patient	Sex	Time Elapsed From Onset of Disease Mos	Bone Maturation Deviation	Eosinophil % Fall
1. CBo	M	6	−0.7	57.1
2. GGe	F	7	+1.7	100.0
3. AHe	F	7	−2.6	22.2
4. BEl	M	8	+0.1	58.3
5. PPo	M	8	+0.3	55.6
6. ECr	F	19	+0.1	40.0
7. BHi	F	21	+0.1	66.7
8. MSc	F	79	−1.7	46.2
9. KSm	F	80	nd	33.3
10. WPe	M	128	+0.3	33.3
		Mean	−0.3	51.3

Table 5. Composition of Patients with Cerebral Palsy in Control Group in Relation to Bone Maturation and Thorn Test Results

Patient	Sex	Bone Maturation Deviation	Eosinophil % Fall
1. RCz	M	nd	44.4
2. PHa	M	−0.2	50.0
3. ECl	F	−1.9	33.3
4. ACo	M	−1.8	45.5
5. JMu	M	−3.4	55.6
	Mean	−2.3	45.8

TABLE 6. COMPOSITION OF PATIENTS WITH CONGENITAL MALFORMATION IN CONTROL GROUP IN RELATION TO BONE MATURATION AND THORN TEST RESULTS

PATIENT	SEX	DIAGNOSIS	BONE MATURATION DEVIATION	EOSINOPHIL % FALL
1. CMa	F	Congenital hip dislocation	0.0	44.4
2. CPi	F	Congenital hip dislocation	−0.7	50.0
3. LEi	F	Congenital hip dislocation	−1.3	33.3
4. DMa	M	Club foot	−1.6	54.5
5. VTh	F	Club foot	nd	55.6
6. MAn	F	Club foot	−0.1	100 0
7. LSe	M	Club foot	−0.2	63.6
8. MNe	F	Pseudarthrosis	nd	58.3
9. RDr	M	Pseudarthrosis	−3.4	33.3
10. PGi	F	Meningomyelocele	+0.5	40 0
11. CCa	F	Meningomyelocele	−1.5	57.1
12. GCo	M	Spina bifida	−0.7	62.5
		Mean	−0.9	54.4

TABLE 7. COMPOSITION OF PATIENTS WITH MISCELLANEOUS DISORDERS IN CONTROL GROUP IN RELATION TO BONE MATURATION AND THORN TEST RESULTS

PATIENT	SEX	DIAGNOSIS	BONE MATURATION DEVIATION	EOSINOPHIL % FALL
1. TJe	F	Traumatic foot deformity	−0.3	62.5
2. RBu	M	Traumatic paraplegia	−0.6	21.4
3. PSl	M	Hemophilic contractures	nd	57.1
4 CSh	M	Chronic metatarsal osteomyelitis	−0 9	23.1
5. MSa	F	Deformity of leg, etiology undetermined	+0.4	46.7
6. CZe	M	Osgood-Schlatter disease	nd	50.0
7 LRi	F	Muscular dystrophy	+2 7	36.4
		Mean	+0.3	42 5

TABLE 8. CONTROL GROUP; THORN TEST AND LENGTH OF HOSPITALIZATION DATA

Patient	Admission Date	Thorn Test Date	Length of Hospitalization Mos.	Eosinophil Counts Fast	4 Hr.	% Fall
1. RC	1-26-56	4- 4-56	2	225	125	44.4
2. CB	10-17-55	3-29-56	5	217	93	57.1
3. CC*	3-14-56	3-27-56	0	217	93	57.1
4. BE	8-24-55	4-12-56	8	300	125	58.3
5. GG*	10- 5-55	4-12-56	6	300	0	100.0
6. PH	10-17-55	3-29-56	5	248	124	50.0
7. TJ*	1- 9-56	4- 6-56	3	200	75	62.5
8. CM*	3-12-56	4-11-56	1	225	125	44.4
9. DM	10-17-55	4-12-56	6	275	125	54.5
10. MN*	9-19-55	4-12-56	7	300	125	58.3
11. VT*	4-23-56	4-23-56	0	225	100	55.6
12. GC	11- 2-55	4-12-56	5	248	93	62.5
13. PHa	2-15-56	3-27-56	1	186	0	100.0
14. KS*	1-11-56	4-12-56	3	150	100	33.3
15. EC*	11-30-55	4-12-56	4	186	124	33.3
16. CP*	12-20-55	5- 8-56	5	250	125	50.0
17. WP	1- 9-56	5- 8-56	4	300	200	33.3
18. RB	4- 6-55	5- 8-56	13	350	275	21.4
19. AC	1-23-56	5-15-56	4	275	150	45.5
20 PS	4-23-56	5- 8-56	0	175	75	57.1
21. RD	8- 5-55	5- 8-56	9	300	200	33.3
22 CS	3-26-56	5- 8-56	1	325	250	23.1
23 LE*	3- 5-56	5-10-56	2	225	150	33.3
24. AH*	11- 2-55	5-10-56	6	225	175	22.2
25. ECr*	11-17-54	5-10-56	18	250	150	40.0
26. LR*	2-13-56	5-10-56	3	275	175	36.4
27. MS*	4- 4-56	5-10-56	1	325	175	46.2
28 BH*	12- 1-55	5-10-56	5	300	100	66.7
29. PG*	2- 6-56	5-10-56	3	250	150	40.0
30. LS	5- 9-56	5-21-56	0	275	100	63.6
31 PP	9-14-55	5-21-56	8	225	100	55.6
32. MNe*	4- 4-56	5-21-56	1	375	200	46.7
33 CZ	4- 9-56	5-21-56	1	300	150	50.0
34. JM	4-11-56	5-27-56	1	225	100	55.6

* Females

TABLE 9. CONTROL GROUP
SEX, CHRONOLOGIC AGE AND BONE AGE DATA

Patient	Sex	Date of Birth	Date of Maturation Plate	Chronologic Age In Mos.	Bone Age In Mos. 1	2	3	Avg.
1. LRi	F	3-10-50	5- 1-56	62	96	90		93
2. MSc	F	9-21-46	4- 4-56	114	94	96		95
3. BHi	F	6-21-46	5-15-56	119	132	120	120	120
4. PGi	F	4-23-47	5-15-56	109	114	114		114
5. LSe	M	12-21-51	5-15-56	53	54	48		51
6. PPo	M	10- 5-46	3-26-56	114	120	114		117
7. MSa	F	7-18-43	3- 6-56	152	156	156		156
8. JMu	M	10-28-49	5-15-56	79	48	48		48
9. CBo	M	2-20-50	6-13-56	76	72	68		70
10. CCa	F	8-30-47	3-14-56	102	94	84	88	86
11. BEl	M	6-25-49	4-20-56	82	72	81	84	83
12. GGe	F	8- 5-52	5-21-56	46	50	60	60	60
13. PHa	M	6- 5-51	5-15-56	59	54	60		57
14. TJe	F	4-30-48	5-31-56	97	94	94		94
15. CMa	F	5- 4-51	6- 6-56	61	69	61	60	61
16. DMa	M	8-23-49	5-15-56	80	66	66		66
17. GCo	M	1-27-43	3- 1-56	157	150	150		150
18. Man	F	12- 5-50	2-15-56	62	60	62		61
19. ECl	F	1-22-47	4-23-56	111	90	90		90
20. CPi	F	12-20-52	5-15-56	41	36	36		36
21. WPe	M	2-16-44	5- 2-56	147	144	156	150	150
22. RBu	M	4- 8-51	6- 4-56	62	54	60		57
23. ACo	M	4-25-42	5-15-56	169	150	150		150
24. RDr	M	11- 3-48	5-31-56	91	60	60		60
25. CSh	M	12-11-40	5- 1-56	185	174	174		174
26. LEi	F	1- 5-51	5- 1-56	64	60	48	50	49
27. AHe	F	2-16-50	5-15-56	75	50	48		49
28. ECr	F	9-21-47	5- 1-56	103	102	106		104
29. PSl*	M	8- 4-40	Not done					
30 KSm*	F	11-12-49	Not done					
31. KTh*	F	9- 3-49	Not done					
32. MNe*	F	6- 2-53	Not done					
33 RCz*	M	2-27-52	Not done					
34. CZe*	M	3-13-41						

* Patients who had Thorn tests but not maturation plates

TABLE 10. CONTROL GROUP
CALCULATION OF STANDARD DEVIATION OF BONE MATURATION

PATIENT	SEX	BONE AGE MOS. A	CHRONOLOGIC AGE MOS. B	DIFFERENCE (A-B) C	SD FACTOR D	MATURATION DEVIATION C D	% EOSINOPHIL FALL
1. LRi	F	93	62	+31	11.41	+2.7	36.4
2. MSc	F	95	114	−19	11.24	−1.7	46.2
3. BHi	F	120	119	+ 1	11.65	+0.1	66.7
4. PGi	F	114	109	+ 5	10.82	+0.5	40.0
5. LSc	M	51	53	− 2	8.08	−0.2	63.6
6. PPo	M	117	114	+ 3	9.39	+0.3	55.6
7. MSa	F	156	152	+ 4	10.53	+0.4	46.7
8. JMu	M	48	79	−31	9.02	−3.4	55.6
9. CBo	M	70	76	− 6	9.08	−0.7	57.1
10. CCa	F	86	102	−16	10.49	−1.5	57.1
11. BEl	M	83	82	+ 1	8.95	+0.1	58.3
12. GGe	F	60	46	+14	8.48	+1.7	100.0
13. PHa	M	57	59	− 2	8.72	−0.2	50.0
14. TJe	F	94	97	− 3	10.27	−0 3	62.5
15. CMa	F	61	61	0	11.77	0.0	44.4
16. DMa	M	66	80	−14	8.99	−1.6	54.5
17. GCo	M	150	157	− 7	10.46	−0.7	62.5
18. MAn	F	61	62	− 1	11.41	−0.1	100.0
19. ECl	F	90	111	−21	10.99	−1.9	33.3
20. CPi	F	36	41	− 5	7.23	−0 7	50 0
21. WPe	M	150	147	+ 3	10.40	+0.3	33.3
22. RBu	M	57	62	− 5	8.85	−0.6	21.4
23. ACo	M	150	169	−19	10.77	−1.8	45.5
24. RDr	M	60	91	−31	9.02	−3.4	33.3
25. CSh	M	174	185	−11	11.96	−0.9	23.1
26. LEi	F	49	64	−15	11.18	−1.3	33.3
27. AHe	F	49	75	−26	10.08	−2.6	22.2
28. ECr	F	104	103	+ 1	10.53	+0.1	40.0

Table 11. Legg-Calvé-Perthes Syndrome Group
Sex, Chronologic Age and Bone Age Data

Patient	Sex	Date of Birth	Date of Maturation Plate	Chronologic Age In Mos.	Bone Age In Mos. 1	2	3	Avg.
1. PLy	M	2-22-53	5-15-56	39	24	30		27
2. MIa	F	4-19-52	5-15-56	49	36	36		36
3. RMa	F	2-10-49	5-15-56	87	78	81		80
4. LRo	F	12-28-46	6- 6-56	113	96	90		93
5. RJa	M	9-19-50	6- 6-56	69	72	72		72
6. TKe	M	5-27-46	5-15-56	120	120	126		123
7. FMa	M	6- 6-48	6- 6-56	96	90	96		93
8. TSh	M	4-20-48	5-15-56	97	84	90		87
9. WSm	M	7- 8-47	5-16-56	78	78			78
10. JSw	M	12-29-50	5-16-56	65	48	48		48
11. JSe	F	9-12-48	5-15-56	92	84	90		87
12. JWo	F	1-23-48	6- 6-56	100	82	94	94	94
13. FBu	M	8-22-48	5- 1-56	92	72	87	66	69
14. JSh	M	12-20-48	6- 6-56	90	60	65		63
15. LHa	M	8-27-47	3-22-56	103	78	72		75
16. JGr	M	9-30-46	3-22-56	114	114	120		117
17. CGa	F	4-27-49	3-22-56	83	66	57	60	59
18. AEl	M	7- 4-46	3-22-56	117	120	129	120	120
19. JDa	M	11-29-47	3-22-56	100	69	81	72	71
20. AFe	F	1-25-51	4-23-56	63	50	45		48
21. GCh	M	2-10-44	3-23-56	145	108	117		113
22. THa	M	7-11-46	3-22-56	116	84	78		81
23. CHo	M	10-24-50	3-22-56	65	57	60		59
24. JO'N	M	6-17-49	6- 6-56	84	69	69		69
25. WOa	M	9-24-46	6- 6-56	116	102	116	116	116
26. KDe	M	8-30-51	3-15-56	52	42	42		42
27. RFa	M	6- 1-47	3-22-56	106	90	93		92
28 JSu	M	2-21-47	6- 6-56	111	54	48		51
29. RHe	M	3-13-48	3-22-56	96	72	81	72	72
30. RPi	M	6- 8-51	6- 6-56	60	54	36	36	36
31. DBu	F	7-18-52	4-23-56	45	30	33		32
32. RGr	M	10-13-50	3-22-56	65	54	51		53
33. JMu	M	5- 7-53	5-15-56	36	24	28		26
34. RWo	M	11-29-51	2-29-56	51	30	30		30
35. MKe	M	2-22-51	6- 7-56	63	63	60		62
36. DTe	M	10- 3-53	2-11-58	52	54	52		53
37. DLo	M	9- 3-53	3- 4-58	54	42	42		42
38. DMcN	M	1-24-51	2- 7-58	84	84	78		81
39. FFa	M	7-12-48	2-24-58	115	116	108	108	108
40. TCh	M	3- 2-52	3- 4-58	72	60	60		60
41. WSt	M	4-28-46	3- 7-58	142	78	84		81
42. RMi	M	10-30-52	2-24-58	64	48	54		51
43 TSt	M	6- 6-52	2-24-58	69	54	60		57
44. JNo	F	4-19-51	1-15-58	81	72	69		71
45. DLaP	F	1- 3-52	2- 4-58	73	46	51		49
46. ALaC	M	3- 5-50	2-11-58	95	60	60		60
47. RWi	M	9-23-48	1- 6-58	111	96	96		96

TABLE 12. LEGG-CALVÉ-PERTHES SYNDROME GROUP
CALCULATION OF STANDARD DEVIATION OF BONE MATURATION

Patient	Sex	Bone Age Mos. A	Chronologic Age Mos. B	Difference (A-B) C	SD Factor D	Maturation Deviation C/D	% Eosinophil Fall
1. PLy	M	27	39	−12	5.24	−2.3	50.0
2. MIa	F	36	49	−13	9.13	−1.4	55.6
3. RMa	F	80	87	− 7	9.92	−0.7	58.3
4. LRo	F	93	113	−20	11.15	−1.8	62.5
5. RJa	M	72	69	+ 3	9.08	+0.3	50.0
6. TKe	M	123	120	+ 3	9.79	+0.3	25.0
7. FMa	M	93	96	− 3	9.10	−0.3	33.3
8. TSh	M	87	97	−10	9.10	−1.1	66.7
9. WSm	M	78	78	0		0.0	50.0
10. JSw	M	48	65	−17	8.95	−1.9	40.0
11. JSe	F	87	92	− 5	10.03	−0.5	40.0
12. JWo	F	94	100	− 6	10.40	−0.6	42.9
13. FBu	M	69	92	−23	9.04	−2.5	42.9
14. JSh	M	63	90	−27	9.01	−3.0	60.0
15. LHa	M	75	103	−28	9.04	−3.1	42.9
16. JGr	M	117	114	+ 3	9.39	+0.3	22.2
17. CGa	F	59	83	−24	9.69	−2.5	25.0
18. AEl	M	120	117	+ 3	9.59	+0.3	14.3
19. JDa	M	71	100	−29	9.07	−3.2	57.1
20. AFe	F	48	63	−15	11.30	−1.3	54.6
21. GCh	M	113	145	−32	10.39	−3.1	16.7
22. THa	M	81	116	−35	9.52	−3.7	37.5
23. CHo	M	59	65	− 6	8.95	−0.7	22.2
24. JO'N	M	69	84	−15	8.91	−1.7	75.0
25. WOa	M	116	116	0		0.0	66.7
26. KDe	M	42	52	−10	7.79	−1.3	50.0
27. RFa	M	92	106	−14	8.98	−1.6	50.0
28. JSu	M	51	111	−60	9.20	−6.5	81.8
29. RHe	M	72	96	−24	9.10	−2.6	75.0
30. RPi	M	36	60	−24	8.79	−2.7	44.4
31. DBu	F	32	45	−13	8.23	−1.6	58.3
32. RGr	M	53	65	−12	8.95	−1.3	27.3
33. JMu	M	26	36	−10	5.08	−2.0	40.0
34. RWo	M	30	51	−21	7.51	−2.8	30.0
35. MKe	M	62	63	− 1	8.89	−0.1	27.3
36. DTe	M	53	52	+ 1	7.79	+0.1	55.3
37. DLo	M	42	54	−12	8.36	−1.4	61.5
38. DMcN	M	81	84	− 3	8.91	−0.3	68.7
39. FFa	M	108	115	− 7	9.46	−0.7	28.8
40. TCh	M	60	72	−12	9.17	−1.3	0.0
41. WSt	M	81	142	−61	10.33	−5.9	37.0
42. RMi	M	51	64	−13	8.92	−1.5	72.5
43. TSt	M	57	69	−12	9.08	−1.3	63.4
44. JNo	F	71	81	−10	9.79	−1.0	14.3
45. DLaP	F	49	73	−24	10.18	−2.4	58.6
46. ALaC	M	60	95	−35	9.08	−3.9	41.0
47. RWi	M	96	111	−15	9.20	−1.6	61.8

TABLE 13. LEGG-CALVÉ-PERTHES SYNDROME GROUP
EOSINOPHIL RESPONSES TO MINOR SURGICAL PROCEDURES *

Patient	Sex	Chronologic Age Mos.	Bone Age Mos.	Maturation SD	Surgery Date	Eosinophil Counts Before Operation	Eosinophil Counts Hours After Operation 4	24	48	72
1. *PLy*	*M*	*39*	27	*−2.3*	*5- 3-56*	*275*	*25*	*125*		*225*
2. RMa	F	87	80	−0.7	4-11-56	200	0	225	200	
3. RGr	M	65	53	−1.3	5- 3-56	225	25	100		225
4. JGr	M	114	117	+0.3	5- 9-56	200	25	75		

* Surgical procedure in all instances was the "determination of direct circulation" of the involved femoral head after the injection of radioactive phosphorus and under general anesthesia. Average duration of procedure was approximately ½ hour.

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Responsa Eosinophilic a ACTH in Relation al Maturation Ossee in Juvenes Stropiate

Summario in Interlingua

Le responsa del numeration eosinophilic al injection intramuscular de ACTH esseva studiate in 81 patientes stropiate de etates de inter 36 e 185 menses. Roentgenogrammas del manos pro le determination del maturation ossee esseva obtenite simultaneemente. Le resultatos esseva dividite in correspondentia con duo gruppos de subjectos con distribution comparabile de etate. (1) Le prime gruppo consisteva de patientes con syndrome de Legg-Calvé-Perthes in qui le maturation ossee esseva cognoscitemente disturbate. (2) Le secunde gruppo serviva de gruppo de controlo e consisteva de subjectos con diagnoses de que disturbation del maturation ossee non es clinicamente characteristic.

Le examine del resultatos del test de Thorn in le duo gruppos revelava in ambes un numero significative de responsas amontante at minus que 50 pro cento. Un analyse additional monstrava un differentia significative in le distribution del procentages de reduction in le numerationes eosinophilic. Le subdivision del gruppo de controlo secundo le diagnoses resultava in le constatation que le resultatos in le casos del patientes de poliomyelitis esseva graphicamente correlationabile con le chronicitate del morbo e etiam con le maturation ossee. Quando il deveniva apparente que un correlation existeva inter le resultatos del tests de Thorn e embe le supra-mentionate factores, le sequente analyses esseva executate: Primo, le relation inter le responsas eosinophilic e le deviationes in le maturation ossee esseva examinate pro le gruppo de controlo in su totalitate per medio de un representation graphic del resultatos individual. Secundo, ille relation esseva examinate per notar le responsas eosinophilic medie como function del deviation medie del maturation ossee occurrente intra complete unitates de intervallo. Iste secunde analyse revelava un correlation linear. Quatro del cinque punctos pro valores medie formava un curva rectilinee.

In le gruppo de patientes con le syndrome de Legg-Calvé-Perthes, le application del mesme technica revelava un nette absentia de correlation con le maturation ossee si ben como con le chronicitate del morbo. (In le caso del presente casuistica, le chronicitate del morbo es adequatemente reflectite in le duration del hospitalisation.) Le graphico del responsas eosinophilic medie pro complete unitates de intervallo in le maturation del ossos indicava que le procentage del reduction in le numeration eosinophilic remaneva relativemente horizontal usque al tempore quando le maturation ossee deveniva minus retardate. Un reduction del responsas medie in le test de Thorn se manifestava con le approache de un positive maturation ossee

Finalmente, certe observationes esseva facite con respecto al responsas del numeration eosinophilic al effecto del trauma de

minor interventiones chirurgic in tres del patientes con syndrome de Legg-Calvé-Perthes. Esseva notate nulle indication de exhaustion o insufficientia adrenal. Le responsas eosinophilic esseva plus tosto disproportionatemente marcate in comparation con le severitate del trauma infligite.

Super le base de iste observationes—i.e. le observation que un correlation existeva inter le responsa eosinophilic a ACTH e le maturation ossee in le gruppo de controlo e le observation additional que nulle tal correlation existeva in le gruppo de subjectos con syndrome de Legg-Calvé-Perthes—le conclusion es formulate que un urgente desiderato ha devenite evidente de investigar additionalmente le function adrenal in relation al maturation ossee con le utilisation de plus precise methodos quantitative in le determination del steroides. Il es probabile que un tal studio va producer importante e significative informationes con respecto al problema del retardate maturation skeletic in casos de syndrome de Legg-Calvé-Perthes.

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Improved X-ray Technic for Hip Fractures*

FREDERIC W. ILFELD, M.D., AND STEPHEN M. FIELD, M.D.

This technic obtains biplane roentgenograms of hip fractures and eliminates exposure of surgical personnel to radiation. The method allows x-ray visualization of the fracture and the position of the nail or pin on both the anteroposterior and the lateral films without moving the leg. It requires no special equipment other than a portable x-ray machine. A gynecologic leg stirrup is used to position the patient on an ordinary operating table and cassette box. A metal book end and a large paper spring clamp hold the x-ray plate for the lateral view (Fig. 1).

Historically, lateral roentgenograms of the hip date back to 1899, when Lauenstein[1] described the flexion and the abduction (frog-leg) position for the lateral view. This method necessitates moving the injured hip and exposes the surgeon to radiation. The first horizontal-lateral (true-lateral) view was described by Lorenz[1] in 1918. It required moving the well leg. This is the basis of our technic. The Clayton Johnson[2] lateral view allows roentgenograms of the injured hip to be taken without moving either hip. However, the technic is rather complicated for surgery, because the x-ray tube must be angled in two directions for the lateral view, and the anteroposterior view does not show the true length of the neck. The caudolateromedial view[1] can be taken with either the x-ray tube or a curved cassette between the legs when a fracture table is used.

METHOD

Before the patient is draped, the normal leg is placed in a gynecologic stirrup, holding the hip and the knee, each flexed to 90°. Reduction of the fractures is done in the routine manner on an ordinary operating table with a Bucky-Potter diaphragm in a cassette box under the pelvis. The reduction can be maintained with a sandbag in a

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FIG. 1. Metal book end (1) supports x-ray cassette (2) in sterile pillow case fastened to sterile drapes with paper clamp (3).



Fig. 2. Note gynecologic stirrup (*1*) elevating well leg, portable x-ray machine (2) and plate holder (3) for lateral roentgenogram of hip.

sterile pillow case on the internally rotated foot.

A portable x-ray machine is placed to take a lateral view of the hip from the far, or normal, side across the table. This is possible, since the well leg is positioned out of the field. The neck of the femur makes an angle of approximately 125° with the shaft. In order that the central ray may be at right angles to the neck, the tube should be set at an angle of 35° with the femoral shaft. The anteversion of the hip is compensated for by the internal rotation of the limb at reduction. Since the films are principally for relationships, the 35° angle can be grossly approximated. (Fig 2)

The x-ray cassette for the lateral view is placed in a sterile pillow case. It is positioned vertically alongside the fractured hip area just above the iliac crest at right angles to the portable central x-ray beam. The cassette is held in place by the metal book end and stabilized with the spring clip holding the sterile pillow case to the drapes. The spring clip has smooth edges.

The x-ray technic for the lateral view is 36-inch distance with a diaphragm on the tube, 75 kilovolts, 3 to 8 seconds, 30 milliamperes, using a grid cassette. The usual anteroposterior view is taken, using an overhead or a second portable machine. The technic is 30-inch distance, 70 kilovolts, 1 to 2 seconds, 30 milliamperes, using a Bucky-Potter diaphragm.

If necessary, one machine may be used. Separate positions are marked on the floor so that the machine can be adjusted easily and rapidly from one position to the next.

EXAMPLES

Case 1. A 48-year-old woman sustained a transcervical fracture of the right hip on October 19, 1958. Two portable x-ray machines were positioned preoperatively. After routine reduction, the Thompson "Z" nail was inserted 2½ inches. Thus, the nail did not reach the fracture site. The roentgenograms were taken of both views without changing the position of the hip, which was maintained by a sandbag on the foot. The roentgenograms showed a good reduction (Fig. 3) However, the nail appeared to be directed posteriorly and possibly would miss the head. Without changing the position of the hip, the nail was withdrawn and the direction was corrected. It was reinserted in a satisfactory position in the head and the neck. Thus, the nail traversed the head and the neck only once, and the hip reduction was maintained throughout (Fig. 4).

Case 2. A 64-year-old woman sustained a transcervical fracture of the right hip on May 20, 1958. It was in good position preoperatively (Fig. 5). The patient had thrombocytopenic purpura A minimum amount of surgery was desirable. At surgery, the same method was employed as in Case 1, except that an overhead x-ray machine was used instead of one of the

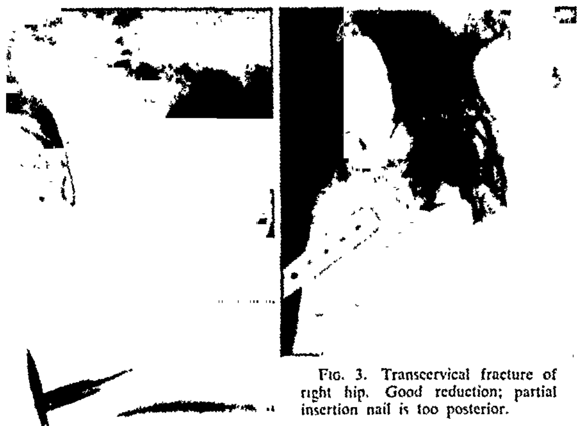


Fig. 3. Transcervical fracture of right hip. Good reduction; partial insertion nail is too posterior.



Fig. 4. Corrected nailing. Nail entered head only one time







FIG. 5. Unstable fracture of neck of right femur.

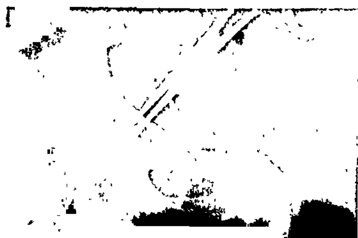




FIG. 6. Partial insertion of pin recorded without moving hip.



Fig. 7 Further insertion of pins

portables. Roentgenograms in both planes were taken to follow the course of the pins as they were inserted through the lateral femoral cortex up the neck into the head. Thus the initial position was not disturbed during the insertion of the pins and the taking of the roentgenograms (Figs. 6-8)

Case 3. A 74-year-old man fell and sustained an intertrochanteric fracture of the right hip on September 25, 1958. He had marked degenerative arthritis of this hip. It was noted during surgery that the frog-leg maneuver would have been impossible, because essentially there was no permissible abduction or rotation of the hip joint. Surgery was performed with an overhead and a portable x-ray machine. (Fig. 9)

DISCUSSION

This procedure is somewhat similar to that used with a fracture table but without its faults. A disadvantage of the fracture table is that the operation is lengthened because the table seldom is ready and the patient has to be positioned on it. Remanipulation of the hip on a fracture table



FIG. 8. Spiral impacted fracture pinn successfully.



is limited because the feet are fastened to the footplates.

In our experience this method of x-ray technic for hip fractures has proven to be simple and time saving. It permits accurate reduction of the fracture. The method allows roentgenograms to be taken in two planes before and after the reduction and partial insertion of the nail or pins. If the fracture is not reduced satisfactorily or the nail is not directed properly, remanipulation and reinsertion of the nail may be done without damaging the head or the neck.

The technic also prevents the possibility c *fragmentation of the head and the neck b* the frog-leg maneuver if the nail is place improperly.

The procedure gives a true lateral roentgenogram of the hip. Therefore, there ca be no error in interpretation of the locatior of the nail, as occasionally occurs when the frog-leg lateral is used.

When the hip has suffered pre-existing disease or trauma, it may be impossible to place the leg in the frog-leg position. In such a case this is the only method by which a lateral view can be obtained.

The book end and the spring clip hold the cassette for the lateral roentgenogram while the foot is stabilized with a sandbag. Since the surgeon does not have to hold the leg for the lateral roentgenogram, he is not subjected to radiation during the x-ray exposure

CONCLUSION

A method of biplane roentgenography for hip fractures has been presented. The book end and the spring clip are simple and effective holders of the lateral x-ray cassette. An ordinary operating table may be used. The fractured leg is not moved. The operating personnel are not exposed to radiation.



FIG. 9. Arthritic hip makes frog-legging impossible. Note position of cone for lateral view.

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Un Meliorate Technica de Radios X pro Fracturas del Coxa

Summario in Interlingua

Le reduction e immobilisation per fixation erne in le tractamento de fracturas del coxa es si ben establite que omne melioration del technica chirurgic debe esser reportate. Le hic-describite methodo permitte le visualisation radioscopic del fractura e del position del clavo in expositiones antero-posterior e lateral sin displaciamento del gamba. Le procedura pote esser effectuate super le tabula operatori conventional con le uso del ordinari porta-pede gynecologic o obstetric, un commun supporta-libros de metallo, e un grande crampa a resorto del typo usate pro crampar papiros.

Le methodo permitte le obtention de radiogrammas in duo planos post le reduction del fractura e le insertion partial del clavo. Si le reduction es imperfecte o si le

clavo es mal placiate, remanipulation e reinsertion del clavo es possibile sin damnificar le capite o le cervice. Isto elimina le insertion multiple de clavos. Illo etiam obvia al periculo de un fragmentation del cervice o capite femoral per le methodo usual a "gamba de rana" in que le coxa es placiate in marcate flexion e abduction.

Le reduction del fractura es effectuate in le maniera routinari super le tabula operatori ordinari con un cassetta con diaphragma de Bucky-Potter sub le pelve. Le gamba normal es placiate in le supporta-pede gynecologic (o su equivalente) con flexion rectangular al coxa e al genu. Un portabile machina de radios X es usate pro le exposition lateral que es obtenite ab latere distal (i.e. le latere del gamba normal). Isto es possibile proque le gamba normal in le porta-pede non obstrue le campo. Le cassetta pro le exposition lateral es deponite in un sterilisate coperi-cossino e placiate verticalmente al longo del exterior del area del coxa fracturate justo supra le cresta iliac in angula recte con respecto al fasce del portabile machina de radios X. Le cassetta es mantenite in le desirate position per medio del supporta-libros de metallo e stabilisate per medio del crampa a resorto que attacha le coperi- cossino al drappos. Proque il non es necessari tener le cassetta manualmente, le chirurgo pote distantiar se ab le campo del radiation X. Le usual exposition anteroposterior es facite per medio de un machina portabile o vertical.

Extense experientias in le uso de iste methodo de roentgenographia in fracturas coxal ha demonstrate que illo es simple e profitabile a causa de su rapiditate. Illo utilisa un tabula operatori ordinari. Illo permitte le accurate reduction del fractura. Illo rende possibile le visualisation del insertion del clavo. Illo elimina le multiple insertiones de clavos. Illo obvia al possibilitate de un fragmentation del capite e del cervice como effecto del manovra a "gamba de rana." Illo evita omne irradiation del chirurgo in le obtention del vista lateral.

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Intertrochanteric Fractures

Duncan C. McKeever, M.D., F.A.C.S.

The fact that intertrochanteric fractures occur mainly in the sixth and the seventh decades, or later, is common knowledge. The general condition of the patient in whom such a fracture occurs has received little attention; and what to do about the condition has received even less. The biomechanics of the injury and its implications in the course of treatment seldom are fully appreciated and often are ignored. Many times intertrochanteric fractures are treated as surgical emergencies when it would be more proper to treat the patient as a medical emergency. The medical emergency treatment recommended in the literature is indefinite and incomplete. The usual long-term convalescent treatment consists of the recommendation of a few vitamin pills, and an adequate diet, not detailed, of which the patient may or may not partake.

The definitive treatment of the fracture, though often carried out skillfully so far as the surgical procedure is concerned, is done without basic knowledge of biomechanics of internal fixation that would ensure successful early union and ambulation in a high percentage of cases. Most surgeons have a single treatment for all intertrochanteric fractures and apply it without considering other methods.

Knowledge of, and careful attention to, the biomechanical principles and the details of the medical treatment of these patients will increase the survival rate significantly. The average patient with an intertrochanteric fracture should be in better physical and mental condition at the end of the healing period than when the incident occurred.

The information necessary to accomplish these ends is available but never has been assembled as a complete therapeutic regimen. Here I will attempt to do this and apply it directly to the problem of the intertrochanteric fracture in order that anyone who has to treat such a fracture can carry it out in complete detail. It is attention to the small details of this treatment that may mean the difference between success and failure, even between life and death.

IMPORTANCE OF THE PATIENT'S NUTRITIONAL STATUS

Usually, the patient who enters the hospital with an intertrochanteric fracture is in a state of poor nutrition and may be extremely malnourished. The older a person gets, the more attention he needs from his relatives, and the less attention he is likely to get. Many of these people live alone and eat a diet consisting largely of cereals, soups, milk and fruit, with a high percentage of carbohydrate, an inadequate amount of proper fats and an extreme deficiency of protein. This may be true even if they are living under the same roof with relatives. Through long habit based on deference to seniority, relatives tend to let the older person, presumably more competent and experienced, take care of himself. They seldom seem to realize that some older person living with them may be eating an entirely inadequate diet that will one day lead to trouble. Frequently the fluid intake of such patients is inadequate. They tend to be constipated. A little inquiry into the patient's habits as to diet and activity, and a casual examina-

tion of the condition of the patient's skin, hair, and nails may reveal that he is living in a state of loving neglect. Suddenly, a fracture occurs, and everyone becomes concerned and wants everything possible done for the patient, though up to that time they have done none of the things that might have avoided the incident. It is probably impossible to educate the general public in the care of the aged. It is even difficult, in most cases, to secure adequate care in the average nursing home and in some hospitals; but there would seem to be no excuse for the physician's not knowing what to do about the patient's general condition once he comes into his hands.

There are patients who are bumped into by other people and knocked down, who are tripped by children or dogs, who stumble on steps or slip on rugs or on icy and wet pavements, and receive direct blows in automobile accidents, especially when the vehicle in which they are riding is hit from the side. Even though these injuries are due to direct trauma, the blow is so slight that in many cases it would not fracture a normal bone.

However, there are many patients who do not fall and break a hip; on the contrary, they break a hip and fall. Some of them may even succeed in holding on to an object of furniture or another person and keep from falling. These fractures are due to indirect trauma. If these people were in a normal state of nutrition and physiology, their bones would not break so easily, and fractures before a fall would occur rarely. Actually, these are fatigue fractures, and the patient has been laying the groundwork for them by glandular malfunction, hormon[illegible] imbalance, poor nutrition and inadeq[illegible] exercises; doing so certainly that suc[illegible] fracture must occur unless some other [illegible] current disease terminates the patient's [illegible] before it happens. There is a gradual [illegible] of calcium in the bones. There is a [illegible] ing of protein [illegible] inally, the cy[illegible] forces become [illegible] in the [illegible] chanteric area, a [illegible] cross-[illegible] density of the bone is decreased to the point at which the stress imposed in taking a step exceeds the elastic limits of the bone in a given area, fracture is certain to occur in the course of ordinary walking. If any unusual strain is applied, it will occur sooner. Frequently, careful questioning of alert patients will reveal that they had fleeting pains of varying severity in the intertrochanteric area for hours, days, even weeks before the bone finally gave way completely.

THE FUNDAMENTAL EMERGENCY AND ITS TREATMENT

I know of no fracture that is in itself an emergency. Any fracture can be treated adequately 2 days, a week, 10 days or more after it is sustained. The end-results will be as good; they may be better. Delay in treatment of the fracture may be lifesaving.

While the fracture is not an emergency, the patient with an intertrochanteric fracture is certainly a medical emergency. It is impossible here to go into detail regarding the reasons for all the treatments or emergency procedures recommended. Some of these ideas are original, some are not. When they are not, an attempt will be made to state their source. Many of them are known if one stops to think, but one should be more interested in the prevention of complications than their treatment after they occur. With sufficient knowledge, foresight and attention to detail, most of them need not occur.

What are the immediate dangers? In the probable order of their importance they are phlebothrombosis, pulmonary congestion, fat embolism and [illegible] All these [illegible]ons and c[illegible] probably are [illegible] within [illegible] hours after the [illegible] though [illegible] may not be [illegible] for [illegible] them, such as [illegible]olism [illegible]ized at all if [illegible] reco[illegible] if he [illegible] to [illegible] the [illegible] us[illegible] and [illegible] th[illegible] given [illegible]

why they are used. Many of these things may sound foolish and time consuming to the family, or even to nurses. Their proper use is important, though it may require considerable insistence on the part of the physician to get them carried out.

The physician will receive a more or less frantic telephone call to the effect that Grandma has fallen and is in great pain, and everyone thinks that she has a broken hip. He replies that he will dispatch an ambulance for the patient at once, and, almost as an afterthought, asks if there is any whisky in the house, or if some can be obtained. He suggests a mixture of 2 ozs. of whisky, 2 teaspoonfuls of sugar and 2 ozs. of water. It may be iced if the patient prefers it. The patient is to sip this until the ambulance arrives. If the patient drinks all of it and wants more, so much the better. The alcohol quiets the nerves, relieves pain, simplifies splinting and transportation, combats shock and, according to Dr. Pipkin, is the earliest possible prophylaxis that one can administer for fat embolism.[4]

One calls the ambulance and gives instructions that the patient be taken directly to the hospital x-ray department. Then a telephone call is made to the x-ray department and instructions are given to slip a plate under the patient's hip and take a portable x-ray on the ambulance cart. A hypodermic may be ordered for pain, though, if the patient has had the whisky, usually this is unnecessary. While someone makes light traction on the leg, the patient is lifted into bed. The clothing is removed. A box one half the width of the bed is placed at the foot of the bed on the uninjured side. The head of the bed then is elevated on blocks at least 15 inches, and preferably 18 inches. Why? This is prophylaxis against phlebothrombosis. I have used it with consistent success for nearly 20 years, since it was first proposed by Frykholm in 1940.[1] If the reader is interested in the reason for its efficacy, he can look up the article, but, as evidence that it does work, I can report that I have not had a postoperative phlebothrombosis or pulmonary embolism in 20 years when this treatment was carried out immediately after operation or after the injury occurred.

With the head of the bed raised and a box put in the foot of the bed on the uninjured side, gravity will apply all the traction necessary to alleviate muscle spasm and keep the patient comfortable. It is my opinion that traction is better omitted, because the average nurse in the average hospital today knows nothing about its proper use and cares less, and, in her hands, it will be more of a hindrance than a help in turning the patients.

Write the following orders, which are expained here as necessary:

1. **Put an overhead frame and trapeze on the bed. Elevate the head of the bed 15 to 18 inches on blocks.**[1]

2. **Insert a Foley catheter.** This avoids a great deal of unnecessary movement and manipulation in women, and precludes retention and residual in men.

3. **Order Azo Gantrisin or other suitable sulfonamide acting on the urinary tract.** There is evidence that usually some infection accompanies the use of indwelling catheters. Hospital orderlies and many nurses are not sufficiently conscious of the dangers of urinary tract infection; nor are they at all consistent in carrying out catheterization under sterile technic. The medication should be continued so long as the Foley catheter is in place and for 2 or 3 days after its removal.

4. **Keep the back rest up a few turns at all times. Change its level a small amount frequently.** This avoids the onset of backache, which may become very distressing, and will usually relieve it if it is present.

5. **Keep a pillow under the knee on the injured side.** This is better than using sandbags. It prevents extreme external rotation and tends to move with the patient. Sandbags do not tend to move with the

patient, and, if the patient shifts position, they may do more harm than good.

6. **With 2 or 3 pillows between the knees, turn the patient on the uninjured side at least 20 minutes 3 times a day. The patient may be turned oftener and stay longer ad lib. While turned, give a thorough back rub, paying particular attention to the sacral area.**

7. **Start at once an intravenous infusion consisting of 1,000 cc. of 5 per cent alcohol with 5 per cent glucose.** This counterbalances the usually decreased fluid intake in the immediate period after injury and continues or initiates the prophylaxis against fat embolism. The alcohol helps to maintain the fat in solution, and the glucose helps to burn it. It is also analgesic.[4] Repeat every 12 hours after injury for 2 days and repeat after surgery for 2 days.

8. **Give intravenously every day 100 mg. of thiamine, 500 mg. of ascorbic acid, 25 mg. of pyridoxine and 100 mg. of niacin. These may be given together. They may be put into other intravenous solutions if such are being given.**

9. **Give 100 mg. of aqueous methyltestosterone immediately and 50 mg. daily intramuscularly for 5 days and twice a week thereafter.** This is given to both males and females.

10. **Vi-estandro (U. S. Vitamin Corp.), 1 Daily.** This starts orally the administration of a balanced dosage of androgenic and estrogenic hormones so essential to protein metabolism and the laying down of a suitable protein matrix in bone formation.

11. **25 mcg. Cytomel Daily.** This is tolerated well even in the presence of cardiac conditions and hypertension, and I give it irrespective of them.

12. **Routine Laboratory Work, Plus a Hematocrit and a Blood Uric Acid.** A drop in hemoglobin is an early evidence of fat embolism.[4] The blood loss, from extravasation, incident to an intertrochanteric fracture may exceed 1,000 cc.

13. **1 Capsule Immediately and 1 Daily of Doxan or Similar Cathartic Accompanied by a Wetting Agent.** This is given routinely. The patient tends to be dehydrated in the first few days. Dehydration helps initiate fecal impaction. In any case, the patient tends to become constipated through the voluntary or the unconscious delay of evacuation. It is better to have the patient err on the side of diarrhea than on that of constipation. Fecal impaction may become a serious complication in the aged.

14. **Give a high protein diet with an adequate amount of fats,** most of which should be in the form of unsaturated vegetable oils. Have the nurse record to what extent the diet was consumed. The caloric intake probably need not exceed 2,000 calories. It is the quality of the intake that is important.

15. **Chart the fluid output and keep it over 1,500 cc. per 24 hours.** Make up the deficiency by administering intravenous fluid in the form of 5 per cent alcohol with 5 per cent glucose. This gives an adequate fluid intake and continues the prophylaxis against fat embolism. If the fluid intake is inadequate, this may be given every 12 hours and at such a rate that it is all administered in not less than 1½ and not over 3 hours. The body cannot metabolize the 50 Gm. of glucose in less than 90 minutes, and, if it is given faster, a portion will be lost through the kidneys. If the solution is given too slowly, the alcohol does not attain sufficient concentration for maximum sedative and analgesic benefit. If it is used, other medication for pain is rarely necessary. If over 3 hours is required, the patient will have been kept in one position too long.

Now one waits. One reassures the patient and the relatives. A tentative date is set for treatment of the fracture 2 or 3 days later, and, if no complications have arisen, definitive treatment of the fracture itself is undertaken. This will necessitate some form of immobilization of the fracture.

METHODS OF FIXATION

If these methods of fixation are to be successful, the selected technic must be carried

out with the same meticulous attention to detail that is necessary in the employment of the prophylactic and the therapeutic medical regimen. The surgeon should be completely familiar with several of these methods of fixation so that the one most suitable to the particular case can be employed. He should be familiar and at ease with the following methods:

1. **Well-leg counter traction**, which *can be used satisfactorily in extremely* senile and debilitated patients. This was described recently in detail by Dr. Herbert Hipps.[2] This method can be applied with local anesthesia, but it does require good nursing care in the postoperative period, with adequate help in handling the patient *in his* general care. The required nursing care almost limits it to hospital or institutional use. This treatment also seems to me to entail an unnecessarily long convalescence. The fracture must heal under constant traction. At best, the compression forces that do so much to promote rapid and firm healing are minimized. In addition to this, rehabilitation is prolonged, and ambulation is difficult after a period in bed or in a wheel chair of 3 months at least with movement restricted by double-leg casts.

2. **Roger Anderson's external skeletal fixation**, the use of which was described recently by Dr. Irvin Scott,[3] is the conservative treatment of choice. Nursing care is simplified, joint motion can be maintained. The only restricted activity is weight-bearing. If the treatment is used properly, union is certain and is sufficiently well advanced for the fixation to be removed in 6 weeks if *ambulation* is not anticipated and in 10 weeks if it is. Ambulation can be started with the fixation still in place. This is an excellent form of treatment if it is carried out in accordance with Dr. Scott's instructions. If this form of treatment is used, it is my opinion that the rate of healing can be accelerated *from* 2 to 4 weeks, provided that the ideal of restoration of anatomic position is abandoned and fixation is applied with the angle between the shaft and the neck at approximately 165° instead of the normal 135° to 140°. This statement is based on the observed healing rate of fractures placed at this angle and on my belief that we do not know the rate at which it is possible for fractures to heal under ideal conditions. We do not even know what all the ideal conditions are or how to set them up. Under certain conditions I have seen intertrochanteric fractures heal sufficiently to *permit full weight-bearing in 6 weeks, and,* at least theoretically, it is possible for such union to occur in 3 weeks.

External fixation can also be applied under local anesthesia, and those who fear infection, drainage or ring sequestration when this treatment is used either have not *used it or do not know how to use it properly.* It is particularly applicable in older individuals and can be used in all cases in which it is possible to use well-leg counter traction. If this treatment is employed properly, the end-results are probably as good as they are with any form of treatment that can be used on intertrochanteric fractures, though there is one form of internal fixation that will bring about more rapid union. External fixation is a form of treatment particularly applicable in obese individuals who are poor operative risks and whose nursing care is so difficult. Within a few days after this fixation is applied, the patient can do everything that he was able to do before the surgery except bear weight on the injured leg.

BLADE PLATES AND NAIL PLATES

In spite of the fact that they have been used in thousands of cases, many times successfully, blade plates and nail plates, slotted or sliding, are not correct biomechanically. The blade or the nail must penetrate the subtrochanteric cortex, traverse the almost hollow intertrochanteric and cervical area, and impinge on or penetrate the extremely *dense head. If it impinges* on this dense portion of the head, the end of the nail or the blade may cut through the relatively thin anterosuperior cervical cortex. If it pene-

trates the dense capital bone, distraction may occur when the screws are applied to the plate and union is certain to be delayed unless the blade or the nail penetrates the head farther. It is difficult for it to do this because of the density of the bone in which it is embedded. If it is driven into the subchondral area, it may penetrate the head and injure the acetabulum. If it does not penetrate the head deeper, union may be so delayed that cyclic stress results in the fracturing of the plate near its juncture with the blade or the nail, or the screws may pull out or break.

If a sliding nail plate or a slotted plate is used, part of this mechanical defect is obviated, but functionally the mechanical gain obtained is offset by the fact that when sliding of the nail or the plate occurs, as it is designed and intended to do, a shearing stress is applied at the fracture line.

METHODS OF TREATMENT

The range of possible complications mentioned above occurs with sufficient frequency to warrant the condemnation of these methods of treatment if one knows something better to do. It is my opinion that there are two methods of treatment superior to any of these devices. These are external skeletal fixation and total intramedullary internal fixation.

A preliminary report on total intramedullary internal fixation will be found in *Clinical Orthopaedics* No. 12.[3] It would seem from the results obtained to date that sufficient union to permit full weight-bearing can be obtained by this method in 6 weeks. At least 1 patient has borne full weight without assistance commencing 2 weeks after the device was inserted. The current design is inserted more easily than a blade plate and increases the angle between the shaft and the neck to approximately 165°, thereby ensuring optimum functional stress on the fracture site and promoting rapid and firm union. Detailed technic of its employment will be reported elsewhere.

All the prophylactic measures listed in the first part of this chapter are just as important, or more so, in the immediate postoperative period and must be carried out with the same attention to detail. The prophylaxis for fat embolism should be reemployed. One must be constantly on the alert for early signs of fat embolism, and, if this complication occurs, it should be treated in accordance with the instructions given by Dr. Pipkin.[4]

During convalescence the patient should lie prone at least twice a day for 20 minutes. The object of this is to prevent flexion contracture that otherwise would occur as a result of the prolonged period of lying down and sitting. If such flexion contracture is permitted to develop, a normal gait is difficult to obtain. If it is not overcome, normal gait never will be attained. The easiest course is to prevent development of this deformity.

Physiotherapy should stress development of abduction and extension power. These also are primary requirements for a normal gait, and these muscles regain normal power and endurance only as a result of exercises.

CONCLUSION

An intertrochanteric fracture is not an emergency, but the patient with an intertrochanteric fracture is an emergency. The surgeon who is to treat him must be responsible personally for the details of his therapeutic and prophylactic medical regimen, and must see that it is carried out properly and completely. He should be thoroughly familiar with two, three or more possible methods of fixation so that the one best suited to the particular case can be employed. Of these available methods of treatment, external skeletal fixation and total intramedullary internal fixation are believed to be the best, for the reasons given. With careful attention to details of the prophylactic and therapeu-

tic medical regimen and the operative procedure, the rate of union can be accelerated significantly, the mortality can be reduced to a fraction of the present accepted percentage, and the average patient should complete his convalescence in better physical and mental condition than when the fracture occurred.

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Fracturas Intertrochanteric

Summario in Interlingua

Fracturas intertrochanteric per se non es occurrentias de urgentia. Sed il ha urgentia in le situation. Il es le patiente con le fractura intertrochanteric que debe esser considerate como un evenimento de urgentia. Le chirurgo qui accepta un tal caso debe facer se personalmente responsabile pro le detalios del regime therapeutic e prophylactic, e ille debe assecurar que omne ille detalios es observate appropriate — e completemente. Le chirurgo debe esser familiar con duo o tres o plus methodos possibile de fixation, a fin que ille pote seliger in omne caso particular le methodo que es adaptate le melio a illo. Es opinate—pro rationes presentate—que inter le methodos currentemente disponibile, le duo meliores es externe fixation skeletic e total fixation interne intramedullari. Le plus precise attention prestate ad detalios del regime prophylactic e therapeutic e del manovra operatori mesme pote resultar in un acceleration del union e in un reduction del mortalitate a un fraction del procentage que es hodie considerate como acceptabile. De facto il es justificate expectar que le patiente typic va completar su reconvalescentia in un condition physic e mental que es superior a su stato al tempore del fractura.

34

The Stone Operation for Hallux Valgus

ROBERT BINGHAM, M.D.

This chapter comprises a paper that was presented at the Western Orthopedic Association meeting held in Portland, Oreg., in October, 1958.

DR. CHARLES A. STONE. I have been doing this procedure for more than 50 years and have performed it more than a thousand times. It is not original with me, although I may have popularized its use to some extent. To my knowledge, details of it have not been published up to now, and I am indebted to Dr. Bingham for this study of his results. Some of my most grateful patients are those who underwent this operation 10, 20 and even 40 years ago. The technic is simple, and the chances of complications and poor results are fewer than in more extensive plastic procedures. I feel highly honored that my name will be associated with this most useful operation.

DR. WARREN WHITE. I came to this meeting chiefly to discuss Dr. Bingham's paper. I cannot recommend the Stone operation too highly. I have been doing it for over 25 years, and the worse the case of hallux valgus or exostosis of the first metatarsal head, the better the result, it seems. I agree with Dr. Stone's routine, and I believe that early walking is the secret of good functional results secured with this procedure over the more technical—and theoretically more anatomic—operations that require prolonged periods of immobilization or restricted weight-bearing after surgery. I have done this operation over 500 times, and I believe it to be the procedure of choice in bunion surgery.

In 1952 Dr. Charles A. Stone, of St. Louis, Mo., diagrammed for me what I consider to be the finest operation yet devised to correct hallux valgus and enlargement of the first metatarsal head in a single procedure. Although he and his associates, Dr. Ralph K. Earp and Dr. Walter P. Graul, have been doing this procedure for many years, Dr. Stone related that he had been "just too busy to sit down and write about it and look up all the literature." My own interest in this problem developed under Dr. Dudley J. Morton.[2]

In the past 6 years I have used the Stone operation for 80 cases of hallux valgus and bunion of the first metatarsal head. The results are so functional and comfortable for the patient that it deserves wider recognition and trial by the orthopaedic branch of the medical profession. The same technic is as successful in hallux rigidus as it is in hallux valgus. Dr. Stone has kindly consented to its publication and contributed to the discussion of this presentation.

As quoted by Bick,[1] hallux rigidus and hallux valgus were first discussed by Davies Colley in 1887. During the last two decades of the 19th century several surgeons advocated resection of a portion of the first metatarsophalangeal joint. Others recommended osteotomy of the first metatarsal. Silver[7] devised a capsulorraphy. In this century some 22 procedures have been described for correction of bunion, hallux valgus and hallux rigidus.

Hemiphalangectomy—the resection of the base of the proximal phalanx of the great toe—was first described by Keller in 1904.[3] This was rediscovered by Brandes in Germany in 1929 and still is the standard procedure of many orthopaedic surgeons. The Keller procedure has several disadvantages. The shortened great toe has a tendency toward dorsiflexion and subluxation. Resection of the exostosis on the first metatarsal necessitates an incision sufficiently long to

operate on both bones of the metatarsophalangeal joint. From ½ to ¾ inch of bone is removed, which causes a very noticeable shortening of the great toe. It also has a 4- to 12-week period of postoperative disability and discomfort.

Hueter, in 1887, and Charles Mayo, in 1905,[4] recommended transverse excision of the head of the first metatarsal just proximal to the exostosis. This operation had the mechanical disadvantage of removing the weight-bearing function of the first metatarsal. It increased pressure on the middle metatarsals of the foot. Frequently patients complained of metatarsalgia and strain of the ankle and the longitudinal arch.

Stone discovered that weight transmitted to the sesamoid bones by the first metatarsal was at the inferior and distal margin of the end of the shaft at the junction with the first metatarsal head. He also reasoned that this weight-bearing margin of the first metatarsal could be preserved and that, at the same time, the enlargement of the metatarsal head, which maintains the hallux valgus deformity, could be removed.

In principle the Stone operation is an oblique resection of the enlarged head of the first metatarsal with preservation of the weight-bearing margin of the metatarsal shaft on the sesamoid bones (Figs. 1 & 2).

Dr. Stone's description of the operative procedure is as follows:[6]

A curved incision is made dorsomedially, beginning just distal to the first metatarsophalangeal joint, approximately 2 inches long. Be sure to keep it about ½ inch medial to the extensor hallucis tendon. Open the joint in the line of the incision. Dissect the capsule laterally beneath the tendon across the lateral side of the head and proximal to the cartilage. Dissect the capsule medially two thirds of the way downward on the head.

Use a curved, medium-sized forceps. Push it under the tendon and down on the lateral side of the neck of the metatarsal. Deliver the metatarsal head through the incision and use a second curved forceps to maintain the position. Use a large double-action bone cutter or osteotome to remove the head in whole or in

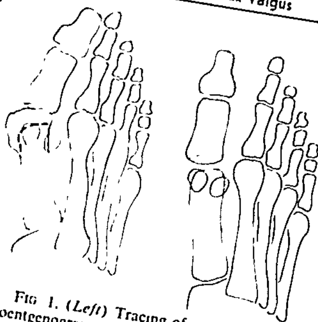


FIG. 1. (*Left*) Tracing of preoperative roentgenogram showing hallux valgus and enlargement of the first metatarsal head of the right foot with metatarsus varus and displacement of the relative normal position of the sesamoids. The broken line shows theoretic line of bone removal in the anteroposterior plane. (*Right*) Tracing of postoperative roentgenographic view of the same patient showing surgical removal of the metatarsal head, correction of the hallux valgus, reduction in metatarsus varus and improvement in weight-bearing positions of the first metatarsal shaft and the sesamoid bones.

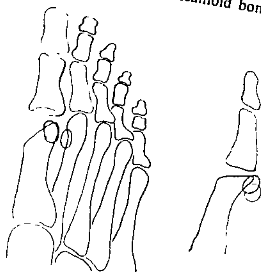


FIG. 2. (*Left*) Oblique tracing of the patient in Figure 1 showing angle of excision of the metatarsal head. (*Right*) Lateral view on the postoperative roentgenogram reveals the width of the pseudarthrotic space and the amount of plantar shift of the first metatarsal remaining in contact with the sesamoids.

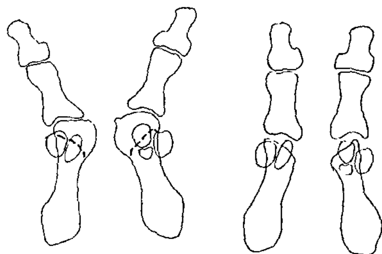


FIG. 3. (*Left*) Preoperative and (*right*) postoperative roentgenographic tracings of a 56-year-old woman with painful degenerative changes in the first metatarsal-phalangeal joints who had comfortable correction of her multiple pathologic conditions by the Stone operation.

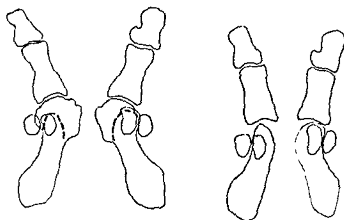


FIG. 4. Roentgenographic tracings of a 66-year-old woman whose chief discomfort was from bunion enlargement of the first metatarsal heads. The Stone operation removed these and improved her hallux valgus deformities.

parts. (Fig. 6, *left*) The bone excised is in two planes, from dorsally downward to the articular margin of the cartilage on the plantar surface of the metatarsal, and from a point just proximal to the exostosis downward to remove it parallel to the medial border of the foot. Nearly ⅜ inch of space should be left between the metatarsal shaft and the phalanx. Round off any sharp edges of the metatarsal. Do not scar the base of the phalanx, although hypertrophic edges can be removed if necessary on the dorsal and the medial margins

Usually, the vein on the dorsomedial surface of the metatarsal is severed Clamp it and tie it with No 000 plain catgut Sew the capsule with a No 00 chromic suture over the head of the metatarsal so as to obliterate the space between the metatarsal neck and the phalanx The fascia can be closed with a few interrupted fine, plain catgut sutures, and the skin closed with a fine dermal lockstitch. A gauze pad is folded and strapped between the second toe and the great toe so that it is in a line or a slightly overcorrected position with the medial border of the foot (Fig 6, *right*) The wound is dressed with fairly thick, soft padding. It may bleed somewhat from the raw bone surfaces. With stockinet, bandage round the foot, then a turn or two round the ankle, and then down between the great and the second toes over the pad Rebandaging may be necessary after a few hours or in a day or two if there should be swelling and discomfort. Elevate the feet with pillows or with the knee-and-foot rest, and apply an icecap to the foot if there is much pain

The patient is allowed out of bed and may walk bearing full weight on his feet whenever he is comfortable, usually on the second or the third postoperative day. Usually he leaves the hospital in 3 to 5 days. Sutures and bandages are left in place for 8 to 10 days When the sutures are removed, a piece of 1-inch felt is bandaged between the first and the second toes for 2 to 3 weeks. Then a small pad is worn between these toes for 4 or 5 months The whole toe of a wide shoe can be cut out, leaving the laces, and tied on loosely for early walking. After about 4 weeks a normal large soft shoe can be worn Later a smaller shoe can be worn if the pads are kept in place. It is wise to order a recheck roentgenogram of each foot in the hospital.

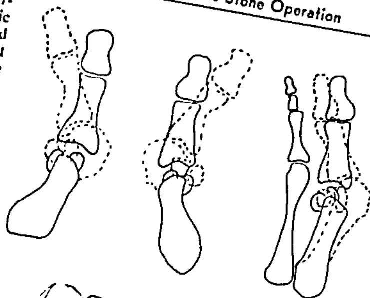


FIG. 5. (*Left*) Superimposed preoperative and postoperative roentgenographic tracings of a 49-year-old clerk who could walk and stand all day with comfort at her job following surgery. Note the change in position of the sesamoids following the bilateral Stone operation. (*Right*) Severe metatarsus varus with painful head enlargement in a 45-year-old woman with good cosmetic and functional result from a unilateral Stone procedure.

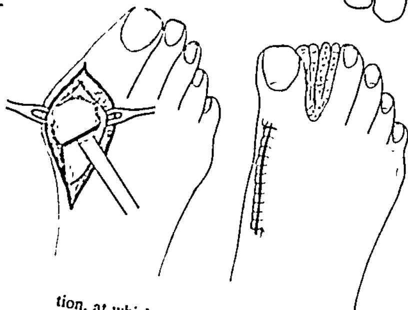


FIG. 6. (*Left*) Oblique removal of the metatarsal head in the anteroposterior view. The plantar cortex remains longer than the dorsal portion of the shaft to bear weight on the sesamoids. The sharp edges of the cortex are rounded off with a rongeur and a curette. (*Right*) Gauze 4 × 4 in. dressing folded twice and placed for bandaging between the great and the second toes following closure of the surgical incision with a running lock suture.

RESULTS OF THE STONE OPERATION

In the past 6½ years I have performed approximately 80 operations on 54 patients, who varied in age from 22 to 89 years. A 93-year-old woman lived an additional 3 years after this surgery enjoying comfortable feet. She told her friends that if she had known "how easy the operation was I would have had it done 20 years earlier."

Operative time varied from 20 to 40 minutes per foot. The average bilateral operating time was approximately 1 hour. The shortest hospital stay was 48 hours; the longest, 14 days. The majority of the patients were hospitalized 5 days. One patient returned to her regular office works 7 days after surgery. The average employed person was off work 3 to 6 weeks. The patients were followed for 3 months after the operation, at which time they were discharged and asked to return if necessary. Only 4 patients came back, and these wanted advice on special shoes. None of the patients had postoperative infections, probably because it is routine practice with me to irrigate the wounds thoroughly before I close them. For this I use dilute penicillin G potassium solution—200,000 units in 100 cc. of saline.

In a follow-up questionnaire, this series of patients all reported themselves as being "improved." Many patients—such as waitresses, nurses, clerks and schoolteachers—have to be on their feet about 8 hours a day. Other than generalized foot fatigue, none complained of discomfort in the operative area. Two patients developed residual stiffness in the metatarsophalangeal joints at site of operation; two patients with rheumatoid arthritis had some increased meta-

tarsalgia; seven others, who complained of metatarsalgia before surgery, claimed that the operation reduced the metatarsal arch *pain by increasing the amount of weight that* they could bear on the first metatarsal and the great toe; three patients were concerned about the cosmetic appearance due to shortening of the great toe. Most patients considered their toes to have been improved in appearance. Many enjoyed wearing new and more stylish types of shoes, although I prescribe low-heel Oxfords routinely.

CONCLUSIONS

The Stone operation is a technical advance for the correction of hallux valgus and bunion by oblique resection of the first metatarsal head. It affords some weight-bearing function of the first metatarsal on the sesamoids. (Figs. 5-6)

The Stone operation is simple in technic, requires a short hospitalization, permits early full weight-bearing and has the shortest *period of disability of any procedure yet* devised for correction of hallux valgus, hallux rigidus and enlargement of the first metatarsal head.

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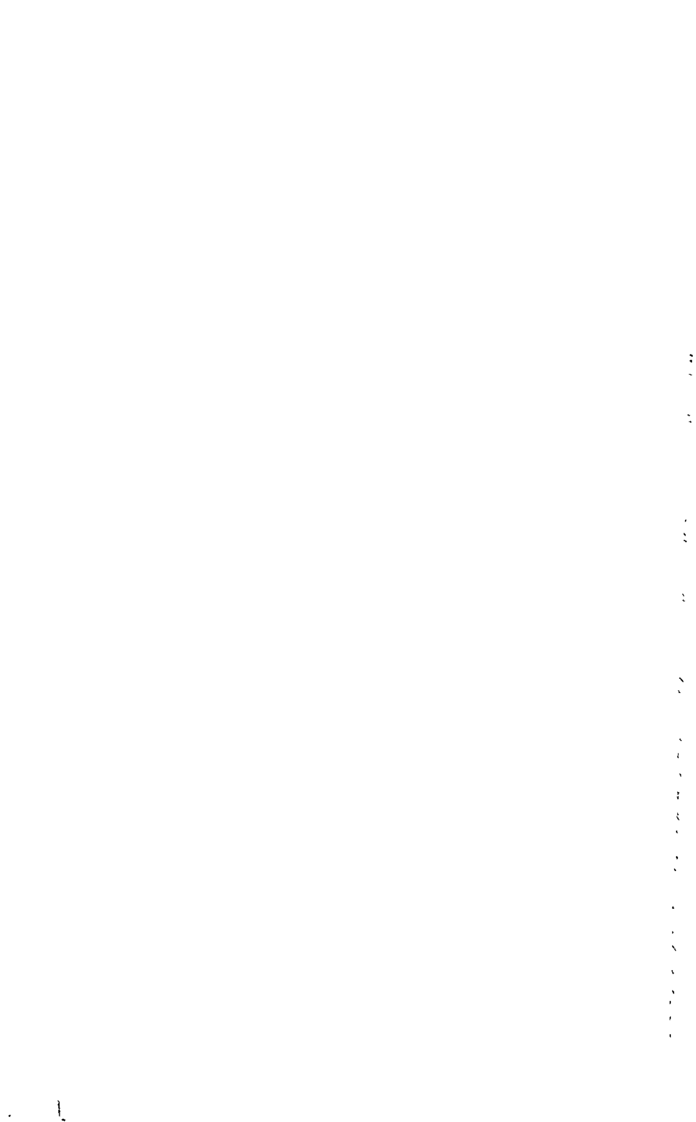
Le Operation Stone pro Halluce Valge

Summario in Interlingua

Es describite un simplissime technica chirurgic pro le correction de halluce valge e de exostosis e allargamento del prime capite metatarsal in un sol manovra. Es presentate le resultatos obtenite in octanta-quatro casos que esseva operate a bon successo per medio de iste technica. Le autor attribue le merito de haber disveloppate le technica a Doctor Charles A. Stone de St. Louis, Missouri, qui ha usate lo in le curso de plus que cinquanta annos in plus que mille casos. In despecto de su utilitate in le preservation del function del halluce e simultaneemente in corriger deformitates e meliorar le apparentia cosmetic, le technica ha non previemente essite describite in le litteratura. Diagrammas monstra le technica de excider le allargate capite metatarsal e de preservar monobstante le function portatori del prime diaphyse metatarsal super le sesamoides. Le methodo original de Doctor Stone es presentate in detalio. Inter le avantages functional del methodo, lo quasi immediate capacitate portatori sin apparatos de supporto debe esser signalate.

Section III

ITEMS



35

Ulnar Artery Thrombosis in the Palm*

A Case Report

ALBERT F. MARTIN, M.D.

Ulnar artery thrombosis in the palm apparently is very rare, only 6 cases having been reported in the literature. Von Rosen[5] published his report in 1934, and subsequent cases were presented by Teece,[4] Jackson[3] and Goren[2] and two cases by Costigan, Riley and Coy.[1] However, the author feels that ulnar artery thrombosis is probably not as rare as the reports would indicate; and, since treatment by excision of the thrombosed segment is so effective in relieving symptoms, an additional case is presented to increase the reader's awareness of the condition.

The ulnar artery passes deep to the volar carpal ligament and lateral to the pisiform bone, and parallels the ulnar nerve that lies adjacent to it on the medial side. The deep ulnar branches of the artery and the nerve arise just distal to the pisiform bone. Then the artery passes superficial to the transverse carpal ligament, and, as it lies on this relatively incompressible structure, it is covered by skin, fat, palmaris brevis muscle and the fascicles of the palmar fascia. It is in this area that ulnar artery thrombosis has been reported, and this is where it occurred in the present case report. In view of the susceptibility of the ulnar artery to trauma by compression as it passes over the transverse carpal ligament, it seems strange that arterial thrombosis is not sustained more frequently.

CASE REPORT

E. L., a male, 32 years of age, was first injured on December 28, 1959, by a .22 caliber gunshot in which the missile fractured the second and the third metacarpal bones of the right hand. Surgical débridement and immobilization resulted in prompt and complete healing, but several fragments of lead remainer adjacent to the base of the third and the fourth metacarpals on the volar surface (Fig. 2). The man returned to his work as a laborer 10 weeks after his initial injury. In May, 1959, he was cutting electrical wiring with pliers and, after

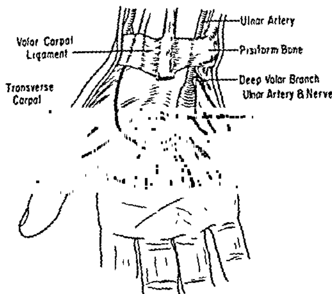


FIG. 1. This line drawing shows the site at which thrombosis of the ulnar artery occurred in the palm. See arrow. (After Costigan, Riley & Coy: J. Bone & Joint Surg. 41-A:702)

* From the Veterans Administration Hospital and the University of Utah School of Medicine, Salt Lake City, Utah.

The author is indebted to Dr. Terence H Cochran for his assistance in preparing the pathologic material used.

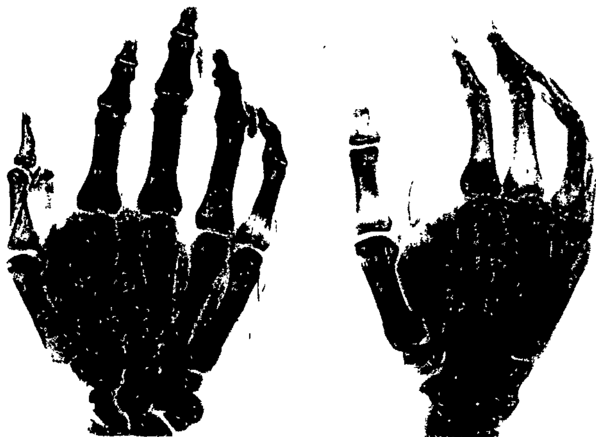


FIG. 2. Preoperative roentgenograms showing the distribution of lead within the hand.

cutting a particularly heavy wire, noticed a dull pain in the hypothenar eminence of the right hand. For 3 weeks this pain worsened progressively, and a tender mass became palpable in this area. Examination revealed a very tender, easily palpable mass just distal to the pisiform bone. This mass was slightly movable and very firm, and it did not pulsate There was neither pallor of the fingers nor any evidence of ulnar neuropathy. The general physical examination was within normal limits, there being no evidence of a systemic arteritis A diagnosis was made of foreign body reaction to the retained palmar lead The man was hospitalized again, and surgical exploration of the hypothenar area was performed under tourniquet control At surgery the palpable mass was found to be a thrombosed segment of the ulnar artery rather than a foreign body The ulnar artery was expanded, thrombosed and surrounded by friable granulation tissue that also was attached to the ulnar nerve The thrombosed section of the artery was removed, and the ends were ligated Then the wound was closed in layers. Postoperative convalescence was excellent, with complete aleviation of pain after incisional healing. The patient received no physiotherapy postoperatively, and after 5 months follow-up has remained asymptomatic.

The segment of artery removed measured .7 by 1.7 cm. Through numerous oblique and transverse sections of the specimen, histologic study revealed organizing thrombus material occluding the lumen of a large artery. There was considerable granulation tissue round the artery. Elastic and connective tissue stains (Verhoeff and Van Gieson) revealed large areas of scarring in the muscular wall, as well as intimal and adventitial fibrosis. In many areas there was loss of continuity of the internal elastica (Fig. 3). Undoubtedly, part of the size and the configuration of the artery was secondary to fibrosis of the artery, with some aneurysmal dilatation. This was interpreted by the pathologist to indicate an arteritis with secondary thrombosis

DISCUSSION

Following ulnar artery thrombosis, there is usually local pain in the hypothenar eminence accompanied by a palpable mass or

fullness in this area. Also there frequently is evidence of ulnar neuropathy, with pain and hypesthesia in the ring and the little fingers. Occasionally pallor of these fingers occurs in cold weather. In the cases reported, diagnosis rarely has been made prior to definitive exploration, and symptomatic treatment has preceded surgery. This has included stellate blocks, local anesthesia, splinting and physiotherapy. All have been futile, no cures having been reported. However, resection of the thrombosed segment has resulted in complete alleviation of symptoms.

The etiology of this form of arterial thrombosis is probably direct trauma. In the

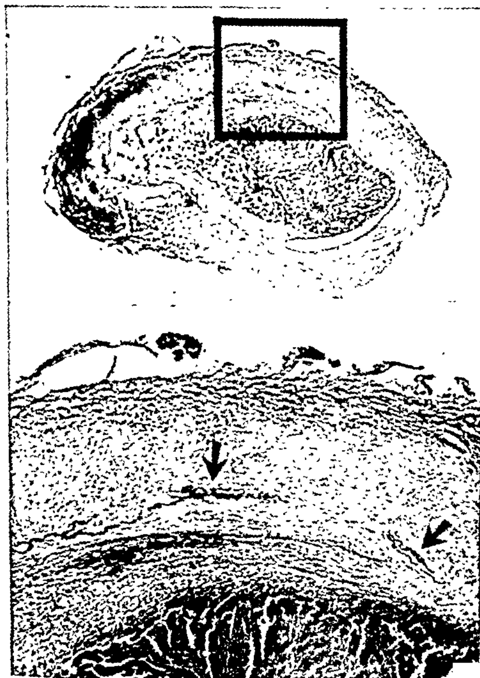


FIG. 3 (*Top*) Verhoeff and Van Gieson stain to show the los internal elastica as well as the occlusion of the lumen of the u artery by organizing thrombus (Magnification × 17) (*Bottom*) Ph micrograph under higher power to demonstrate further the los internal elastica. (× 78) Arrow points to internal elastica.

previous cases reported, as in this one, there was evidence of injury to the area of the hypothenar eminence. In this case, compression of the ulnar artery by the plier handles is probably of greater significance than the presence of the bullet fragments in producing the ulnar artery thrombosis. Von Rosen reasoned, as is believed, currently that aneurysm formation was more likely to follow damage to the media of the artery wall, whereas thrombosis usually followed intimal injury. In the case described above, elements of both are present.

SUMMARY

A case of ulnar artery thrombosis in the palm is presented. The thrombosis was probably secondary to direct trauma. Complete relief of pain and return to gainful employment followed simple resection of the thrombosed segment of the artery. An awareness of this entity should increase the incidence of diagnosis and provide the basis for early definitive treatment.

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36

Successful Frederick Thompson Hip Prosthesis in a Patient 107 Years Old*

MARIO PALAFOX, M.D., LOUIS W. BRECK, M.D., AND EMMANUEL CASIANO, M.D.

For some years we have been using, with satisfactory results, the Frederick Thompson hip prosthesis in all those cases in which it was indicated and the patient's general health permitted it. Age per se has been no contraindication to its use.

Our principal indications for using the hip prosthesis in fresh fractures are the following: an unstable fracture, a vertical fracture of the femoral neck that cannot be locked, any fractures that cannot be reduced satisfactorily, severe osteoporosis where it is believed that internal fixation will not hold, and, in general, whenever it is felt that a nailing is impractical.

The woman under discussion here had lived to the remarkably old age of 107 and appeared to have no specific physical defects to contraindicate a surgical procedure. Because of the type of fracture and the severe degree of osteoporosis, a femoral head replacement was considered to be a more practical approach to the problem than an internal fixation.

CASE REPORT

Mrs T. G was first seen on March 26, 1957. Her chief complaint was that of pain in the right hip for the past 2 days. The history was obtained from her daughter, who stated that her mother, who was 107 years of age, had fallen 2 days before in getting out of bed. Since then she had been unable to walk. Immediately after being seen, she was sent to the hospital.

Physical examination on admission revealed an elderly Spanish-American female, apparently in no distress, lying quietly in bed. Her blood pressure was 136/70, pulse 80 and respiration 16. The right hip was held in adduction and external rotation. There was pain on palpation or motion of the hip and some apparent shortening. Additional positive findings were bilateral cataract, heart enlarged to the left, uterine prolapse Grade 3 with cystocele, and right elbow extension limitation due to an old injury.

Laboratory findings were as follows: red blood count, 3,760,000; hemoglobin, 12.4 Gm.; white blood count, 7,700; urinalysis, within normal limits; blood urea nitrogen, 9.0 mg.%. Her electrocardiogram was essentially normal. The radiograph of her right hip showed a subcapital fracture of the neck of the femur with displacement. There was severe osteoporosis of all the bones shown in the roentgenogram.

On the second day after admission, surgery was performed under spinal anesthesia. A posterior Gibson approach was used, and the femoral head was replaced by a Thompson prosthesis. The operative procedure lasted 40 minutes, and the patient withstood it well.

Recovery from the operation was uneventful. The patient was able to sit in a chair on the 2nd postoperative day. She was kept on a sheepskin to prevent decubitus. She was fitted with a pessary by a gynecologist to correct the procidentia. She was discharged from the hospital on April 6, 1957, her 9th postoperative day, by ambulance in good condition. On April 10, 1957, the sutures were removed, and the incision was found to have healed well. Roentgenograms of the right hip, taken on

* From The El Paso Orthopaedic Surgery Group.



FIG. 1. Radiograph of hip of patient 3 months after the prosthesis was inserted.



FIG. 2. The patient 2 years and 3 months after operation—at this time 109 years of age.

May 1, 1957, revealed the prosthesis to be in excellent position (Fig 1). At that time the patient was allowed to begin walking with the help of her daughter. She has been seen at regular intervals since and has been doing very well. She was last seen on June 24, 1959, 2 years and 3 months after her operation, at the age of 109, and then, too, was found to be doing well (Fig 2).

COMMENT

The reader may be interested in what substantiation there is of the patient's age of 107 at the time of operation On May 28, 1956, the *El Paso Herald-Post* ran an article on its front page on Mrs. Tirsa Gonzalez when she was 106. It had made some investigation of the matter and was satisfied as to her claim. She was born on a ranch in Durango, and this, according to her parents, was in 1849. She came to the United States from Torreón in 1914 and was widowed in 1919. In the newspaper article referred to, Mrs. Gonzalez made the following statement:

I must have been 13 when the French invaded Mexico. [The French invaded Mexico in 1862.] They didn't bother us in our small ranch near Lerdo, so I never saw them, but I remember the joy I got from being shown where a lot of the invaders fell from Mexican fire. Those Frenchmen went into Mexico like balls of fire but left with their tails burning.

The patient's father lived to the age of 95 and her mother to 80. The patient had 6 children, 4 boys and 2 girls; 3 survive.

Concerning the success of the hip prosthesis operation, it can be said that the patient was made comfortable and active by this operative procedure. It might very well be that if she had not been operated on, the confinement to bed necessitated by the hip fracture would have caused her death. At the present time she is alive and well at the age of 109. So far as we have been able to determine, this is the oldest patient on whom a Frederick Thompson hip prosthesis operation has been performed successfully.

SUMMARY

A case is reported in which a Frederick Thompson hip prosthesis was placed in a patient aged 107. The patient survived and has been doing well for over 2 years.

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